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Cooperative Oncogenesis and Polyploidization in Human Cancers: A Dissertation

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A Dissertation Presented

By

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COOPERATIVE ONCOGENESIS AND POLYPLOIDIZATION IN HUMAN CANCERS

A Dissertation Presented

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DEDICATION

I would like to dedicate this work to my grandfather Murray Rubin. While growing up, he instilled a sense of the importance of education in me. He was always very proud of me and encouraged me to do well, but still have fun, in school. I know that my achievement here would have made him so happy and proud. Although he has been gone a long time there is no one that I would rather share this PhD dissertation with than my grandfather.

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Lastly, I must thank my husband Destin. I have nothing but the utmost respect for his scientific intelligence, which has helped me immensely over the years. And I truly appreciate and am grateful for his loving soul, which so obviously matches mine.

ABSTRACT

A common phenotype observed in most cancers is chromosomal instability. This includes both structural and numerical chromosomal aberrations, which can promote carcinogenesis. The fusion gene *CBFB/MYH11* is created by the structural chromosomal inversion(16)(p13.1q22), resulting in the fusion protein CBFβ-SMMHC, which blocks differentiation in hematopoietic progenitor cells. This mutation alone, however, is not sufficient for transformation, and at least one additional cooperating mutation is necessary.

The role of wildtype *Cbfb* in modulating the oncogenic function of the fusion protein Cbfβ-SMMHC in mice was examined. Transgenic mice expressing the fusion protein, but lacking a wild-type copy of *Cbfb,* were created to model the effects of these combined mutations. It was found that wild-type *Cbfb* is necessary for maintaining normal hematopoietic differentiation. Consequently, complete loss of wild-type *Cbfb* accelerates leukemogenesis in *Cbfb/MYH11* mice compared to mice expressing both the fusion and wild-type proteins. While there is no evidence in human patient samples that loss of wild-type *Cbfb* expression cooperates with the fusion protein to cause transformation, it is apparent from these experiments that wild-type Cbfβ does play a role in maintaining genomic integrity in the presence of Cbfβ-SMMHC. Experiments have also shown that loss of *Cbfb* leads to accumulation of hematopoietic progenitor cells, which may acquire additional cooperating mutations.

Not unlike *CBFB/MYH11*, the human papillomavirus (HPV) E6 and E7 proteins are not sufficient for cellular transformation. Instead, high risk HPV E7 causes numerical chromosomal aberrations, which can lead to accumulation of additional cooperating

mutations. Expression of HPV-16 E7 and subsequent downregulation of the retinoblastoma protein (Rb) has been shown to induce polyploidy in human keratinocytes. Polyploidy predisposes cells to aneuploidy and can eventually lead to transformation in HPV positive cells.

There are several possible mechanisms through which E7 may lead to polyploidization, including abrogation of the spindle assembly checkpoint, cleavage failure, abrogation of the postmitotic checkpoint, and re-replication. Rb-defective mouse and human cells were found to undergo normal mitosis and complete cytokinesis. Furthermore, DNA re-replication was not found to be a major mechanism to polyploidization in HPV-E7 cells upon microtubule disruption. Interestingly, upon prolonged mitotic arrest, cells were found to adapt to the spindle assembly checkpoint and halt in a G1-like state with 4C DNA content. This post-mitotic checkpoint is abrogated by E7-induced Rb-downregulation leading to S-phase induction and polyploidy.

This dissertation explores two examples of the multi-step pathway in human cancers. While certain genes or genetic mutations are often characteristic of specific cancers, those mutations are often not sufficient for transformation. The genetic or chromosomal abnormalities that they produce often stimulate the additional mutations necessary for oncogenesis. The studies with *Cbfb/MYH11* and HPV E7 further exemplify the significance of numerical and structural chromosomal aberrations in multi-step carcinogenesis.

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PREFACE

The work contained in this thesis is represented in the following publications:

Heilman SA, Nordberg JJ, Liu YW, Sluder G, Chen JJ. HPV-E7 induces polyploidy through abrogation of a novel post-mitotic checkpoint. *Manuscript in submission*.

Heilman SA, Kuo YH, Goudswaard CC, Valk PJM, Castilla LH. The role of *Cbfb* in *Cbfb/MYH11* associated leukemogenesis. *Cancer Research*. 2006. 66:11214-8.

Additional unrelated studies performed in fulfillment of the Ph.D. degree will not be presented in this thesis and are represented in the following publications:

Liu YW, **Heilman SA**, Illanes D, Sluder G, Chen JJ. Abrogation of a Postmitotic Checkpoint Contributes to Human Papillomavirus E6-induced Polyploidy in Human Keratinocytes. *Cancer Research*. 2007.

Kuo Y-H, Landrette SF, **Heilman SA**, Perrat PN, Garrett L, Liu PP, LeBeau MM, Kogan SC, Castilla LH. Cbfb-SMMHC induces distinct abnormal myeloid progenitors able to develop acute myeloid leukemia in the mouse. *Cancer Cell*. 2006. 9: 57-68.

Castilla, LH, Perrat P, Martinez NJ, Landrette SF, Keys R, Oikemus S, Flanegan J, **Heilman S**, Garrett L, Dutra A, Anderson S, Pihan GA, Wolff L, Liu PP. Identification of genes that synergize with Cbfb-MYH11 in the pathogenesis of acute myeloid leukemia. *PNAS*. 2004. 101:4924-4929.

CHAPTER I

GENERAL INTRODUCTION

1.1 Chromosomal Instability and Cancer. Cancer is a multistep process involving mutations in multiple genes and genetic pathways usually affecting two major classes of cancer-related genes: oncogenes and tumor suppressors. Oncogenes promote neoplastic transformation when they are aberrantly activated. For example, activation of an oncogene may result in a cellular proliferative advantage. Conversely, tumor suppressors normally function to inhibit transformation, and mutations in them often negate their normal protective function. Whereas oncogenes are often genetically dominant, mutations in tumor suppressors are often recessive. Cells containing both types of these mutations have a significant growth advantage and may expand to form a clonal pre-malignant neoplasm. Additional somatic mutations are necessary for full transformation and progression to malignant and metastatic cancers (reviewed in (Hahn & Weinberg, 2002)).

There are over 100 types of cancers recognized and classified, including both solid tumors and cancers of the blood (leukemias). Most cancers exhibit phenotypic hallmarks that result in genetic mutations in common pathways necessary for transformation. Normal cells undergo senescence or apoptosis after a finite number of divisions, however, transformed cells become immortalized and have unlimited replicative ability (Newbold & Overell, 1983). Immortalization and transformation is often accomplished through mutation of genes that confer proliferative or anti-apoptotic advantages. Cancerous cells are also less responsive to their environment. Most transformed cells exhibit a loss of contact inhibition and do not halt proliferation when touching neighboring cells (Holley & Kiernan, 1968). Other hallmarks include loss of growth factor dependence (Cross & Dexter, 1991) and an ability to grow in the absence

of anchorage (Reddig & Juliano, 2005). A more recently acknowledged hallmark of human cancers is chromosomal instability (CIN), which may accelerate the activation of proto-oncogenes, increase the accumulation of mutations in tumor suppressor genes, and/or facilitate resistance to apoptosis (reviewed in (Lengauer et al, 1998)). CIN may be caused by subtle point mutations or drastic chromosomal aberrations, such as translocations between chromosomes. These more severe mutations can be classified into numerical or structural chromosomal changes.

Numerical chromosomal abnormalities occur when there are changes in the overall number of chromosomes, such as polyploidy and aneuploidy, the state of having an even multiple or an abnormal multiple of chromosomes, respectively. Cells with these types of chromosomal aberrations often produce more mutations as a result of the changing genomic state of the cell (Andreassen et al, 2001; Meraldi et al, 2002). For example, in pre-cancerous Barrett's esophagus cells, polyploidy subsequently leads to aneuploidy, which has been shown to be an early event in multi-step carcinogenesis (Galipeau et al, 1996).

Structural changes are specific rearrangements of chromosomes including deletions, inversions, and translocations. For example, the first structural chromosomal rearrangements to be associated with a specific disease is the translocation t(9;22) also called the Philadelphia chromosome, which codes for the fusion gene *BCR-ABL* (Nowell & Hungerford, 1960). The dimerization activity of the Bcr protein results in constitutive activation of the Abl tyrosine kinase, which creates an oncogene found in all patients with chronic myeloid leukemia (CML) (reviewed in (Barnes & Melo, 2002). Structural

chromosomal aberrations are primarily found in leukemic cells, and many subtypes of leukemia can be classified according to the resulting chromosomal rearrangement.

1.2.1 Acute Myeloid Leukemia. Hematopoiesis is the process by which red and white blood cells are formed in the bone marrow. Proper production of these cells is necessary to defend against infection, protect against injury, and to deliver oxygen. Cells are formed from pluripotent hematopoietic progenitor stem cells through mechanisms of proliferation and differentiation. Cytokine signaling pathways are important for maintaining proper expansion of the blood cells, while transcription factors are important in regulating the differentiation potential of these cells. Tight control over both these processes is important for hematopoiesis and prevention of leukemia. A block in differentiation prevents proper expansion of necessary blood and immune cells throughout the body. An increase in proliferation or a lack of apoptosis increases the amount of immature hematopoietic cells in the body, which may preclude any normal blood cells from carrying out their normal processes.

When a progenitor cell acquires a mutation that results in a block in differentiation, and the same cell also acquires a proliferative or survival advantage, that cell may become transformed. This pre-leukemic cell can then clonally expand and form a leukemic population. Therefore, mutations in both of the differentiation and proliferation pathways are necessary in order to develop leukemia (Speck & Gilliland, 2002). Based on these observations, Gilliland (2001) has proposed a “two-hit model,” where two disparate but cooperating mutations are necessary for leukemogenesis. A “class I” mutation confers a growth advantage to hematopoietic progenitors, including an

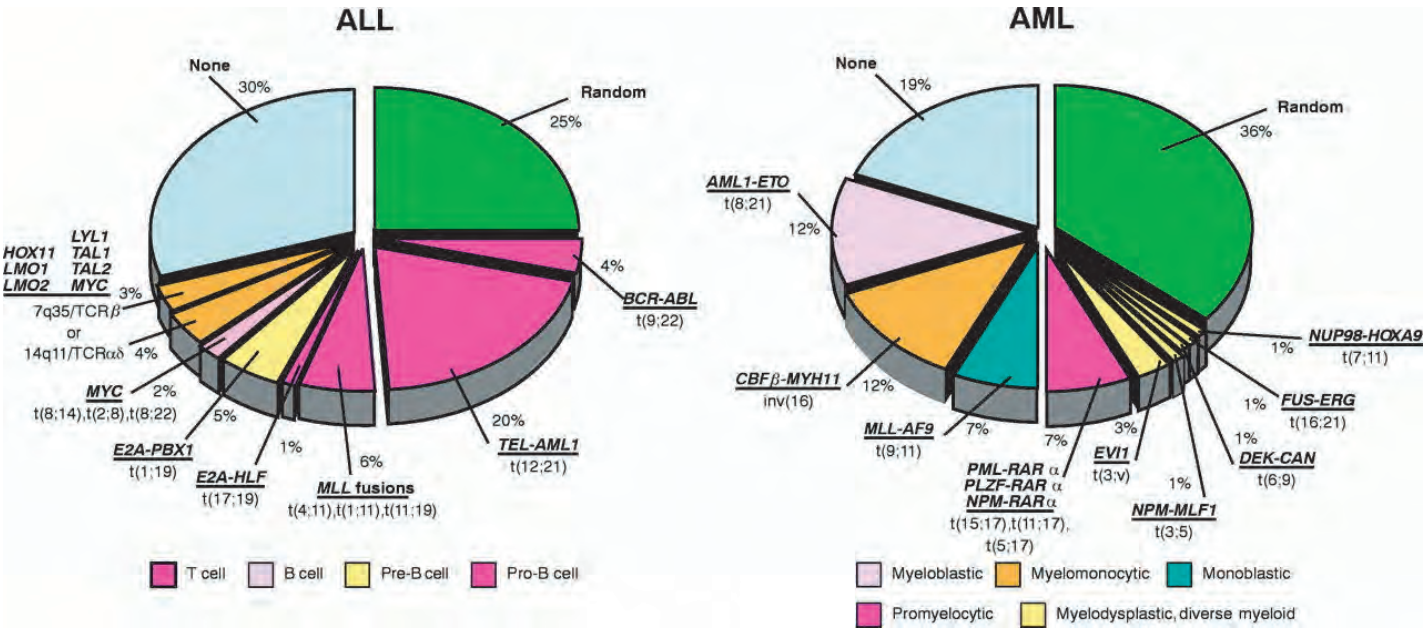
increase in proliferation, self-renewal, or anti-apoptotic function, while a “class II” mutation interferes with hematopoietic differentiation (Gilliland, 2001). The labels “I” and “II” do not imply a temporal order for these mutations, and it is currently unknown as to whether the order of accumulation is important for leukemogenesis.

Clinical analysis of human leukemia patients has shown that a large percentage have non-random chromosomal aberrations, including translocations, inversions, and deletions (Look, 1997). CML is exclusively associated with the aforementioned Philadelphia chromosome t(9;22) (Barnes & Melo, 2002). Genomic aberrations have been detected in over 80% of chronic lymphoid leukemia (CLL) patients including the 13q14, 11q, and 17p deletions (Stilgenbauer et al, 2002). Approximately 65% of acute leukemias contain disease-specific chromosomal translocations. Most of the structural rearrangements in the acute leukemias create oncogenic fusion proteins mainly involving hematopoietic transcription factors important for hematopoietic differentiation and are usually classified as Class II mutations. The cooperating Class I mutations are most often found in oncogenes or tumor suppressors important in apoptosis, proliferation, or the cell cycle.

The acute leukemias include acute myeloid leukemia (AML) and acute lymphoblastic leukemia (ALL), which affect the myeloid and lymphoid lineages respectively. The French-American-British (FAB) system of classification divides AML into 8 subclasses (M0-M7) based on morphology, immunophenotyping, and histochemical staining. The most commonly targeted transcription factor in human AML, affecting 24%, is the Core-Binding Factor (CBF) (Look, 1997) (Figure 1.1).

Figure 1.1. Distribution of chromosomal aberrations in acute leukemias. Acute leukemias are often characterized by distinct chromosomal aberrations including translocations and inversions. The CBF transcription factor is affected in nearly a quarter of acute myeloid leukemias including the fusions *AML1/ETO* and *CBFB*/*MYH11*.

Figure 1.1



(Look 1997)

1.2.2 Core Binding Factor. CBF is a heterodimeric transcription factor important in hematopoietic differentiation. The dimer is comprised of one of three DNA-binding α-subunits (RUNX1, RUNX2, or RUNX3) and a β-subunit (CBFβ)[1]. The RUNX subunits bind the DNA at a specific TGYGGT consensus binding site through the Runt homology domain, which is very similar to the Drosophila Runt protein (Bae et al, 1994). The RUNX subunit also binds to CBFβ through the Runt domain, which causes a conformational change that increases the RUNX binding affinity to DNA (Goger et al, 1999). The RUNX subunit is found primarily in the nucleus (Kanno et al, 1998). Because CBFβ is mostly cytoplasmic (Brown et al, 1995), the β-subunit must be bound to the α-subunit in order to enter the nucleus and act as the CBF transcription factor.

Studies in the mouse have shown that the Cbfβ:Runx1 heterodimer acts as an important regulator of definitive hematopoiesis in embryos, the process through which the fetal yolk sac and subsequently the fetal liver form the erythroid and myeloid lineages of hematopoietic cells in the embryo (Castilla et al, 1996; Okuda et al, 1996; Sasaki et al, 1996; Wang et al, 1996b). The Cbfβ:Runx1 heterodimer also regulates myeloid and lymphoid differentiation in adults (Ichikawa et al, 2004). The CBF complex regulates these processes through transcription of genes such as T-cell receptor β (TCRβ) and interleukin-3 (IL-3) (Cameron et al, 1994; Prosser et al, 1992). The Cbfβ:Runx2 heterodimer plays an essential role in osteogenesis (Komori et al, 1997), and the Cbfb:Runx3 heterodimer is involved in the neurogenesis of the dorsal root ganglia, T-cell differentiation, and maintenance of the gastric epithelium (Ito & Miyazono, 2003).

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[1] Other names for the α and β subunits of CBF: RUNX1= AML1, CBFA2, PEBP2αB. RUNX2= AML3, CBFA1, PEBP2αA. RUNX3= AML2, CBFA3, PEBP2αC. CBFB= PEBP2β.

As previously mentioned, studies in the mouse have determined that both *Cbfb* and *Runx1* are important in definitive hematopoiesis, since *Cbfb*$^{-/-}$ or *Runx1*$^{-/-}$ embryos fail to develop embryonic definitive hematopoiesis and die at midgestation, e11.5-e13.5, due to hemorrhaging in the central nervous system (Okuda et al, 1996; Sasaki et al, 1996; Wang et al, 1996a; Wang et al, 1996b). This phenotype was rescued in *Cbfb*$^{-/-}$ mice by expressing *Cbfb* from the hematopoietic specific promoters *Tie2* or *GATA1* (Miller et al, 2002; Yoshida et al, 2002), further underlining the key role of *Cbfb* during hematopoietic differentiation. It has also been suggested that Cbfβ is essential for the generation of hematopoietic stem and progenitor cells and that decreased Cbfβ expression may lead to an accumulation of these progenitor cells as represented by an increase in c-kit^{+} and/or Sca1^{+} progenitor cells (Kundu & Liu, 2003).

Mutations affecting CBF, including point mutations, translocations, and inversions, are also present in 30% of all human leukemias (Lutterbach et al, 1999; Roulston et al, 1998; Speck & Terryl, 1995), primarily AML and ALL, and also myelodysplasia, abnormal formation of bone marrow cells. However, while point mutations in RUNX1 have been associated with AML (Preudhomme et al, 2000; Reilly, 2005), a correlation between point mutations in CBFβ and AML has not been established (Leroy et al, 2002). The translocation t(8;21)(q22;q22), creating the fusion gene *RUNX1/ETO,* and the inversion inv(16)(p13;q22), creating the fusion gene *CBFB/MYH11,* are the most common chromosomal aberrations in AML (Figure 1.1) (Look, 1997).

1.2.3 CBFB/MYH11. The most common form of the inv16 *CBFB/MYH11* mutation breaks and joins the first five exons of *CBFB,* including the complete RUNX heterodimerization domain, with the C-terminal half of *MYH11,* encoding the smooth muscle myosin heavy chain (SMMHC) protein (Liu et al, 1993). The resulting CBFβ-SMMHC fusion protein can multimerize through the SMMHC multimerization domain and retains its ability to bind to the RUNX subunit (Figure 1.2) (Liu et al, 1993).

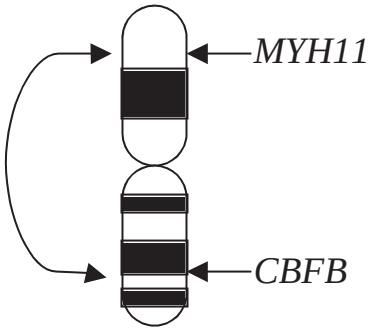
The inv16 chormosomal aberration is tightly associated with AML M4Eo, which is classified as having an increased amount of myeloblasts and abnormal eosinophils in the peripheral blood. Inv16 AML patients also show a significant increase in their white blood cell count and splenomegaly, an enlarged spleen, resulting from the increased difficulty in filtering the increased amount of myeloblasts in the blood.

In order to study the fusion protein, a knock-in mouse model was created (Figure 1.3A). Like the *Cbfb*$^{-/-}$ and *Runx1*$^{-/-}$ embryos, these heterozygous mice also died at midgestation due to failure of definitive hematopoiesis (Castilla et al, 1996; Okuda et al, 1996; Sasaki et al, 1996; Wang et al, 1996a; Wang et al, 1996b). Because only one allele of the fusion gene was necessary for this phenotype, this suggests that Cbfβ-SMMHC is a dominant inhibitor of CBF function.

There are two major models that attempt to explain the dominant CBFβ-SMMHC function over wildtype CBFβ. The sequestration model proposes that the fusion protein binds to the Runx subunit and sequesters it in the cytoplasm, either by forming multimers or by binding to the actin filaments (Adya et al, 1998; Kanno et al, 1998). The repression model proposes that the fusion protein is able to suppress CBF transcription through

Figure 1.2. The *CBFB/MYH11* fusion gene results from an inversion of chromosome 16. The fusion protein CBFβ-SMMHC retains the CBFα heterodimerization domain and the SMMHC coiled-coil multimerization region.

Figure 1.2


MYH11
CBFB

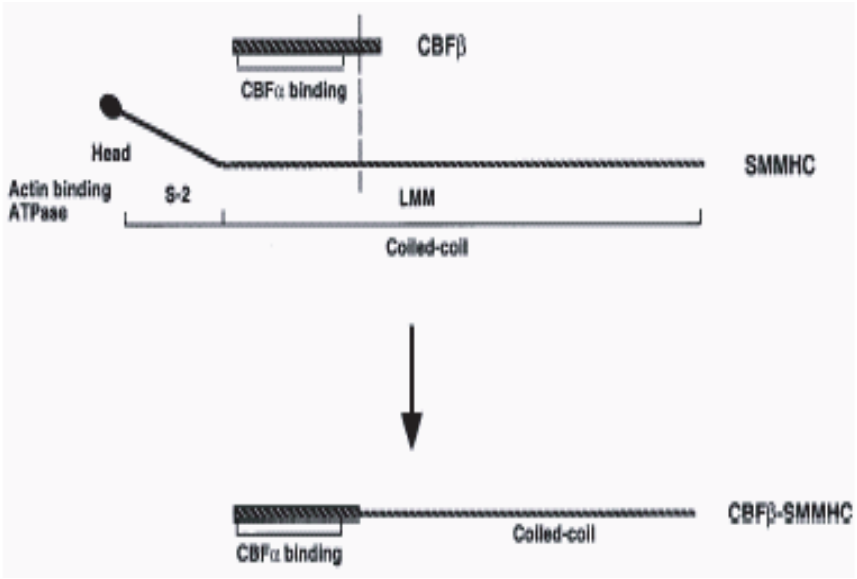
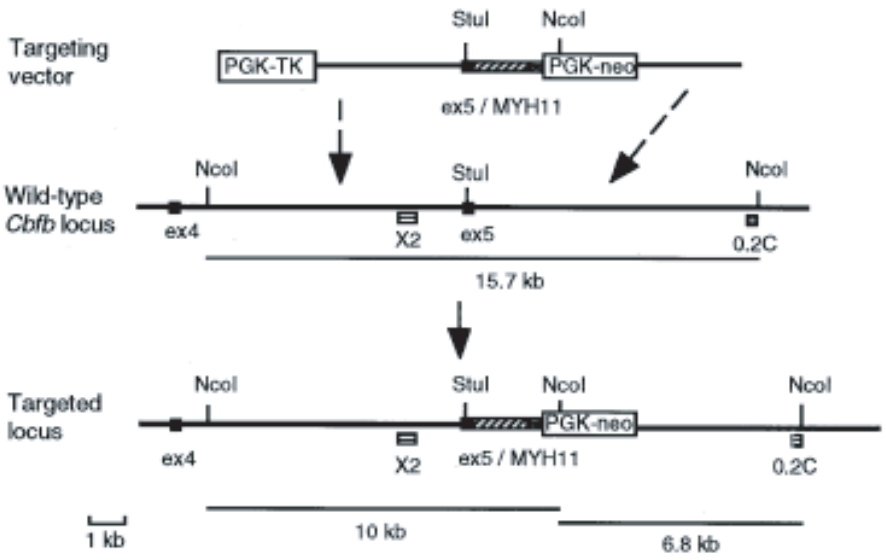
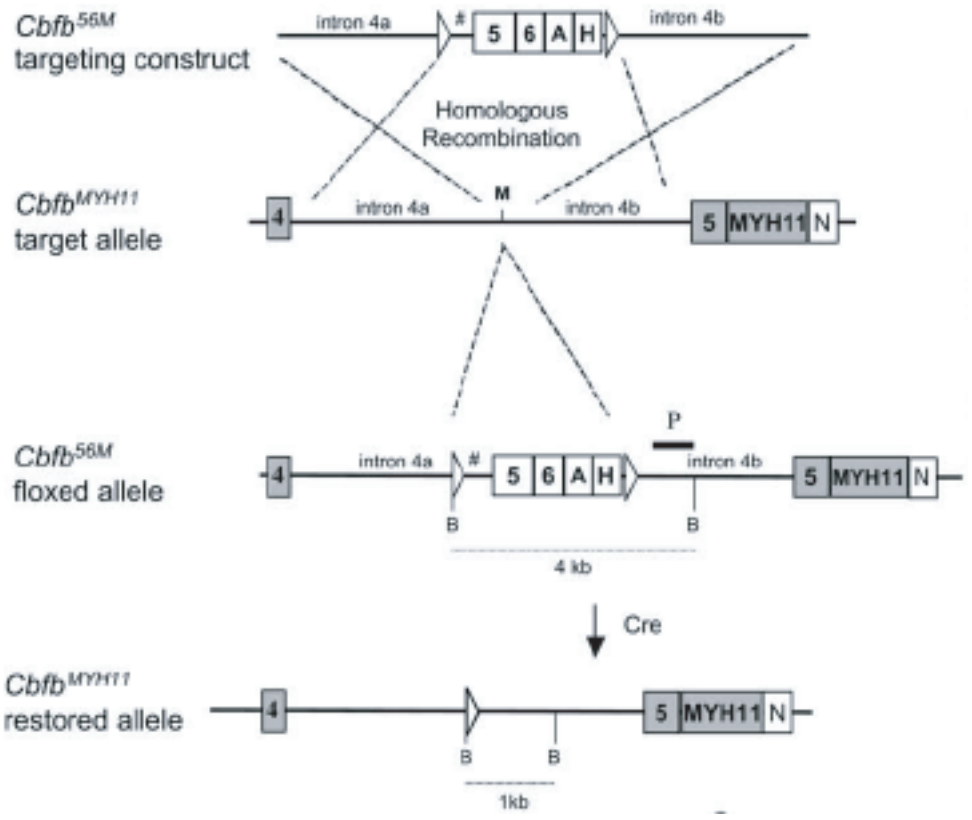

CBFβ
CBFα binding
Head
SMMHC
Actin binding
ATPase
S-2
LMM
Coiled-coil
CBFβ-SMMHC
Coiled-coil
CBFα binding

Figure 1.3. *Cbfb/MYH11* knock-in and conditional knock-in mouse targeting scheme. A. The human *CBFB* cDNA for exon 5 fused to *MYH11* was knocked-into the mouse *Cbfb* gene after exon 4. **B.** The human *CBFB* cDNA for exons 5 and 6 flanked by LoxP sites was inserted into the knock-in gene. Transcription of this gene results in full-length wild-type Cbfβ protein expression. Upon excision of the loxP sites, the fusion gene *Cbfb/MYH11* is transcribed.

Figure 1.3



(Castilla et al, 1996)



(Kuo et al, 2006)

interaction with the repression domain at its C-terminal end, which can bind to AML-1B and recruit corepressors (i.e. mSin3A) to the DNA (Lutterbach et al, 1999).

Isothermal titration calorimetry results suggest that the CBFβ-SMMHC protein disrupts Runx1-CBFβ function by binding to the Runt domain of Runx1 with a higher affinity than wild-type CBFβ (Lukasik et al, 2002). Both CBFβ and SMMHC are able to bind to the Runt domain, and this simultaneous interaction is what causes the higher binding affinity. However, in the presence of DNA, CBFβ and CBFβ-SMMHC bind to the Runt domain with similar affinities. These results provide some explanations for both dominance models. First, the data suggest that the CBFβ-SMMHC fusion protein could sequester Runx1 in the cytoplasm and prevent translocation to the nucleus for CBF transcription. This calorimetry data also supports the repression model by explaining that the Runx subunit could bind to the fusion protein preferentially and result in repression rather than bind to CBFβ and result in normal transcription (Lukasik et al, 2002).

1.2.4 Additional Mutations. Because the knock-in mice are embryonic lethal, chimeras and a conditional knock-in mouse model were developed to study the fusion gene in adults. The targeting scheme for the generation of the conditional knock-in mice uses the Cre-Lox system and is depicted in Figure 1.3B. In this system, *Cbfb* sequences for exons 5 and 6 flanked by loxP sites are inserted into intron 4 of *Cbfb* before the knock-in sequence. This design allows for the full length wild-type *Cbfb* transcript to be expressed in developing mice. These mice were then crossed with Mx1-Cre mice, where Cre is expressed from the interferon responsive Mx1 promoter. Upon addition of polyinosinic-polycytidylic acid (pIpC), which mimics double stranded RNA, the interferon cascade is

activated and Cre is expressed from the Mx1/Cre transgene. This causes excision of the sequences between the loxP sites, exons 5 and 6, and the fusion gene is once again expressed.

Using chimeras and conditional knock-in mice, it has been shown that the fusion protein impairs adult hematopoiesis by causing a partial differentiation block of the myeloid and lymphoid lineages (Castilla et al, 1999; Castilla et al, 1996; Kuo et al, 2006). However, induction of Cbfβ-SMMHC expression or Runx1-loss in adult bone marrow (BM) does not seem to affect the maintenance of long-term hematopoietic stem cells (HSC) (Growney et al, 2005; Ichikawa et al, 2004; Kuo et al, 2006).

Several lines of evidence suggest that Cbfβ-SMMHC may exert an incomplete block of CBF function. First, ectopic expression of the fusion protein in embryonic stem cells expressing one or both copies of *Cbfb* does not inhibit differentiation *in vitro* (Miller et al, 2001). Second, although RUNX1 is important for stem cell emergence, $Cbfb^{+/MYH11}$ knock-in hematopoietic stem cells expressing Cbfβ-SMMHC still persist in the BM of chimeras (Castilla et al, 1999). Last, retroviral insertional mutagenesis in *Cbfb-MYH11* knock-in chimeras identified common insertions in the *Runx2* gene (Castilla et al, 2004), suggesting that Cbfβ-SMMHC leukemic function is affected by levels of Runx proteins.

The chimera and conditional knock-in studies have also shown that while the fusion protein is necessary for leukemogenesis, it is not sufficient, and additional genetic mutations are required for full transformation in these mice (Castilla et al, 1999; Castilla et al, 2004; Kuo et al, 2006). The fusion gene *Cbfb/MYH11* acts as a Class II mutation and therefore requires a cooperating Class I mutation. Cbfβ-SMMHC expression reduces

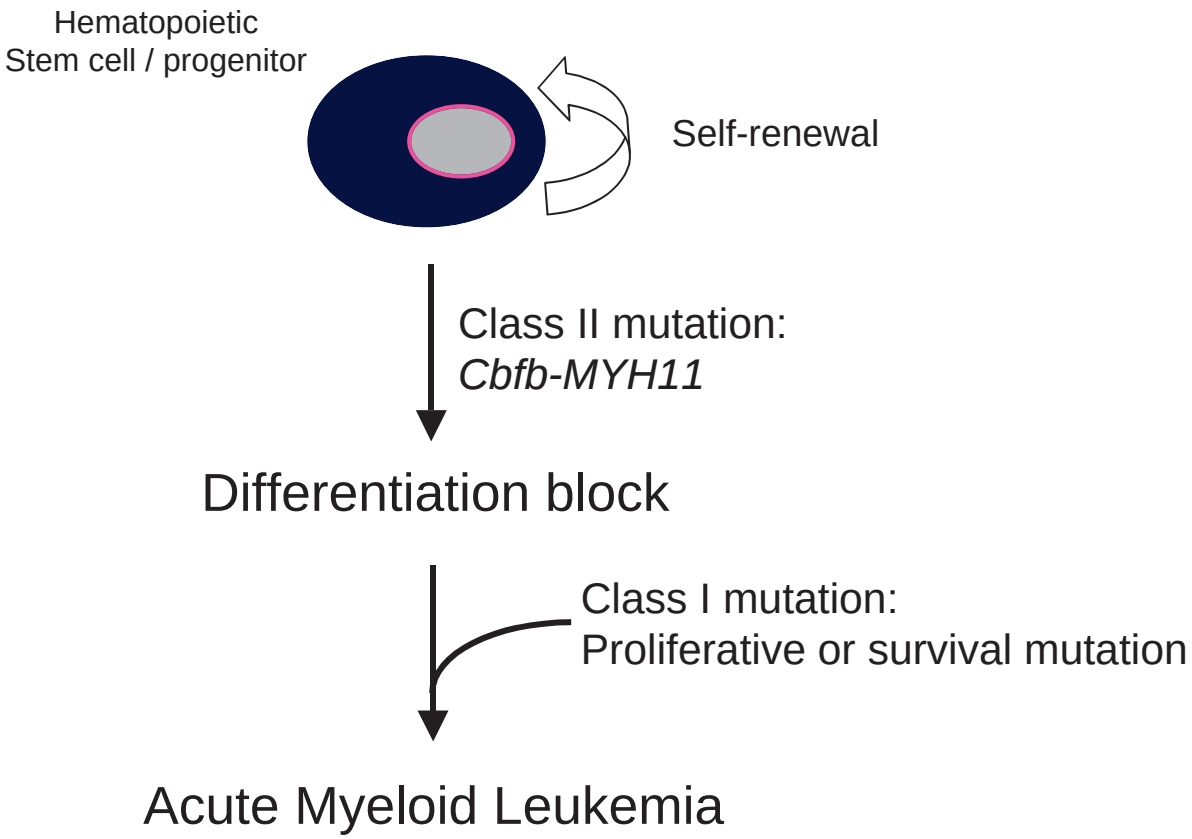
HSC function by inhibiting multilineage repopulation (blocking differentiation) and creating a myeloid progenitor predisposed to leukemia development. (Figure 1.4)

There are several methods of generating and screening for cooperating Class I mutations in *Cbfb/MYH11* mice. A common method to generate many mutations is through addition of N-ethyl-N-nitrosourea (ENU), a potent DNA alkylating agent. Although this results in quick onset of leukemia, it is difficult to map the potential contributing mutations. Another more practical method is retroviral insertional mutagenesis (RIM), which was performed in our lab by infecting *Cbfb/MYH11* mice with the amphotropic murine leukemia virus (MLV) strain 4070A. The virus is able to insert itself throughout the genome and induce misexpression of genes in one of several ways. The virus may integrate upstream of a gene's transcription initiation site or in the 5'UTR where the strong viral promoter and enhancer sequences present in the long terminal repeat (LTR) would cause promoter activation and upregulation of the gene, most likely an oncogene. Alternatively, the virus may insert in the coding region of a gene resulting in a truncated or disrupted protein, such as a tumor suppressor. In those mice that then develop leukemia, inverse PCR can be used to map the locations of these insertions, thereby determining the potential disruption that acted as a cooperating class I mutation. Finally, a candidate based approach can be used where mutations in common oncogenes or tumor suppressors are tested for cooperation with the *Cbfb/MYH11* fusion to cause leukemia.

Class I mutations that have been shown to correlate with *Cbfb/MYH11*-induced leukemias include activating mutations in the receptor tyrosine kinases, c-KIT and FLT3, and the RAS genes, specifically N-RAS and K-RAS (Reilly, 2005). However, these

Figure 1.4. HSCs require a Class I and Class II mutation for leukemogenesis. The presence of the fusion protein Cbfβ-SMMHC blocks differentiation, however an additional Class I mutation is required for acute myeloid leukemia progression.

Figure 1.4


Hematopoietic
Stem cell / progenitor
Self-renewal
Class II mutation:
Cbfb-MYH11
Differentiation block
Class I mutation:
Proliferative or survival mutation
Acute Myeloid Leukemia

account for less than half of all human *CBFB/MYH11* AMLs. Evidence has also suggested other potential "second hits." These include inactivation of tumor suppressors including p53 or activation of oncogenes such as Plag1 and PlagL2 (Castilla et al, 2004). Landrette et al have confirmed that these oncogenes, aberrantly activated, do indeed cooperate with *Cbfb/MYH11* to form leukemia (Landrette et al, 2005). Also, mutation of the *Runx2* gene has been proposed as a potential cooperating mutation with *Cbfb/MYH11* from RIM where the virus caused truncated forms of RUNX2 to be expressed with a putative dominant negative function (Castilla et al, 2004).

Chapter II of this dissertation tests the hypothesis that Cbfβ modulates the effect of Cbfβ-SMMHC in the BM hematopoietic progenitors and leukemogenesis. Specifically, the study explores whether Cbfβ-SMMHC, a Class II mutation, causes an incomplete differentiation block dependent on Cbfβ levels, and whether the complete lack of remaining wild-type Cbfβ leads to an acceleration of leukemia compared to those mice with the presence of the fusion protein and wild-type Cbfβ. To test this hypothesis, adult myeloid differentiation and leukemia development in mice with a conditional *Cbfb/MYH11* knock-in allele and a *Cbfb* null allele was studied.

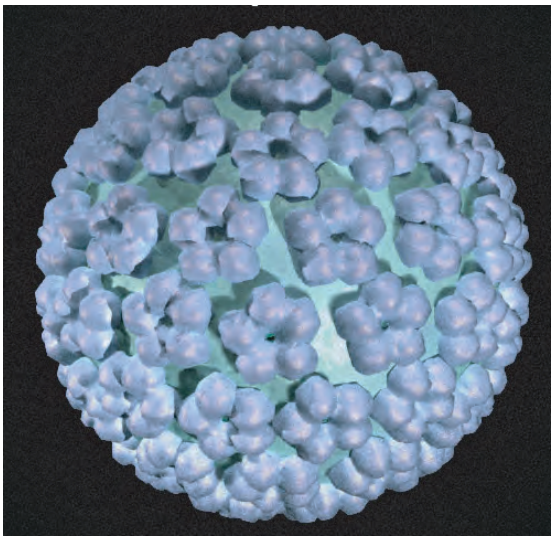
1.3.1 Human papillomavirus. Introduction of viral oncogenes may also act as cooperating Class I mutations. For example, the HPV-16 E7 oncogene has been shown to cooperate with Cbfβ-SMMHC to induce AML in mice. It is thought that E7 does this by accelerating the progression from G1 to S by degrading pRb (Yang et al, 2002).

Papillomaviruses (Figure 1.5A) are small DNA viruses can only infect replicating epithelial cells. Only those cells at the basal epithelium are replicating, and HPV

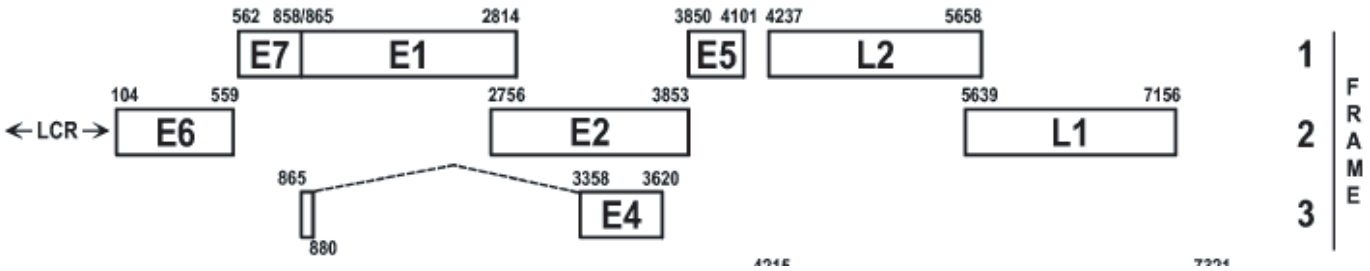
Figure 1.5. Human Papillomavirus. A. Image of HPV capsid proteins. **B.** The HPV genome has 8 open reading frames. These genes code for early (E) non-structural regulatory proteins and late (L) structural capsid proteins.

Figure 1.5

A



B



(Zheng and Baker, 2006)

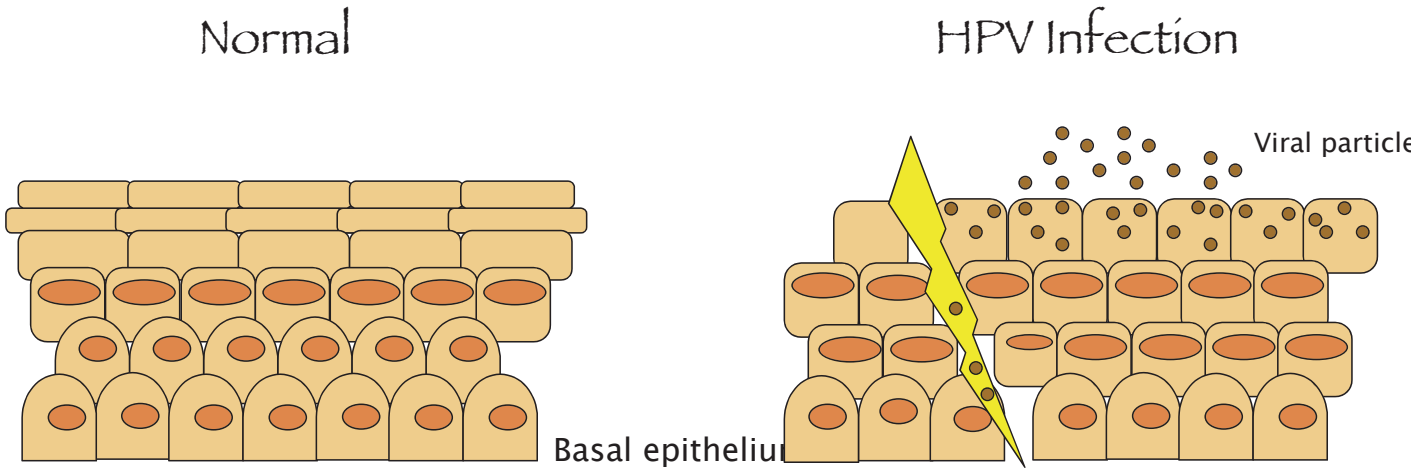
particles reach this lower stratum through micro-lesions and tears in the epithelium. As the basal cells migrate towards the surface, they terminally differentiate and exit the cell cycle. However HPV, like other DNA viruses, must utilize the host cell's replication machinery as it progresses through S-phase. Therefore HPV has evolved proteins that promote cell cycle progression in differentiated cells. As these cells migrate towards the surface, the viral particles are encapsulated and the resulting virions are released to infect other cells (Figure 1.6, reviewed in (Fehrmann & Laimins, 2003).

The HPV genome is a circular double stranded DNA genome consisting of approximately 8 kb. There are more than 100 types of HPV identified, and they infect the genital tract, head, neck, hands, and feet. Those that infect the genital tract are divided into high-risk and low-risk based upon their ability to cause malignant versus benign lesions, respectively. Low-risk HPVs (e.g. types 6 and 11) are primarily associated with benign lesions such as genital warts. High-risk HPVs (e.g. types 16, 18, and 31) are associated with lesions that can lead to high-grade cervical intraepithelial neoplasia and, ultimately, to cervical carcinoma. Cervical cancer is one of the leading causes of cancer death in women worldwide (zur Hausen, 2002), and greater than 99% of all cervical cancers are HPV positive (Walboomers et al, 1999). Worldwide, approximately 493,000 new cases are diagnosed each year, and over 273,000 women die from invasive cervical cancer each year (National Cervical Cancer Coalition).

In addition to cervical cancers, more than 50% of other anogenital cancers and 25% of oral/esophageal cancers are HPV positive (reviewed in (zur Hausen, 2002)). Although tobacco and alcohol are responsible for most head and neck squamous cell carcinomas (HNSCC), there is evidence for a causal association between HPV and a

Figure 1.6. HPV Pathology. HPV infects the basal epithelial layer. As the cells migrate towards the surface, the HPV genome is replicated and encapsulated, and the viral particles are released.

Figure 1.6



subset of HNSCCs (Gillison et al, 2000). Also, HPV-associated cervical and anogenital cancers and HNSCCs are frequently found among HIV-infected individuals because of their suppressed immune system (reviewed in (Del Mistro & Chieco Bianchi, 2001). Their compromised immune systems make their cancers difficult to treat, therefore understanding the precursors of HPV-induced cancers is fundamentally important.

Progress has recently been made in the prevention of HPV-associated diseases in the form of a preventative vaccine against the most harmful types of the virus. In the summer of 2006, the first prophylactic vaccine was approved for prevention of HPV infection. Gardasil® (Merck & Co) is a quadrivalent vaccine which contains recombinant virus-like particles assembled from the L1 capsid proteins of four HPV types- 16 and 18, which together cause 70% of cervical cancers, and 6 and 11, which together cause 90% of genital warts (Villa et al, 2005). Because these particles do not contain HPV DNA, they are not harmful. However the multiple capsid proteins are able to trigger an antibody response in the host to prevent against future HPV-6, 11, 16, or 18 infection.

Unfortunately, this vaccine is not a cure; it does not alleviate pre-existing infections. Also, this vaccine only works on 4 types of HPV, albeit the most potentially harmful, whereas there are over 30 types that are able to infect the genital epithelium. Furthermore, while trials have been promising, the total efficacy in preventing cervical cancer has not been proven over a long period of time (Hymel, 2006). Lastly, vaccinations do not work well in immunocompromised individuals, such as AIDS patients, who are more at risk than the average population for acquiring an HPV

infection. Therefore, elucidating the mechanism behind HPV transformation is still important for the development of therapies against HPV-associated malignancies.

The HPV genome (Figure 1.5B) contains 8 open reading frames, with their gene products divided into two groups, early (E) non-structural regulatory proteins and late (L) structural capsid proteins. The E1 helicase is essential for viral DNA replication (reviewed in (Doorbar, 2006)). E1 cooperates with the E2 transcription factor, which is essential not just for viral DNA replication, but also genome segregation and regulation of early transcription, including that of E6 and E7 (reviewed in (Doorbar, 2006)). The role of E4 is still not fully established, but it has been suggested to play a role in viral late functions (Peh et al, 2004). E5 encodes a membrane protein whose function is unknown but appears to have some transforming activity and is therefore a possible oncogene (reviewed in (Doorbar, 2006). The main transforming properties of high-risk HPVs reside primarily in the E6 and E7 oncogenes and will be discussed further. The two late genes L1 and L2 encode capsid proteins and are important in viral efficiency (reviewed in (Doorbar, 2006)).

1.3.2 E6 and E7 oncoproteins. Because of the importance of the p53 and retinoblastoma (Rb) tumor suppressors in maintaining cellular homeostasis, most DNA tumor viruses have evolved proteins that are able to bind to and inactivate them. For example, Adenovirus E1A and HPV E7 are able to bind to and inhibit Rb. This prevents Rb's interaction with E2F, leaving the E2F transcription factor free to activate S-phase proteins thereby causing progression of the cell cycle. Proteins from these same viruses, Adenovirus E1B55k and HPV E6, are able to bind and inhibit p53 function as well. The

Simian Virus 40 (SV40) Large T protein is able to bind and inactivate both Rb and p53. In most of these viruses, including HPV E6 and E7, the proteins not only facilitate replication of the virus, but often lead to transformation of the host cell as well.

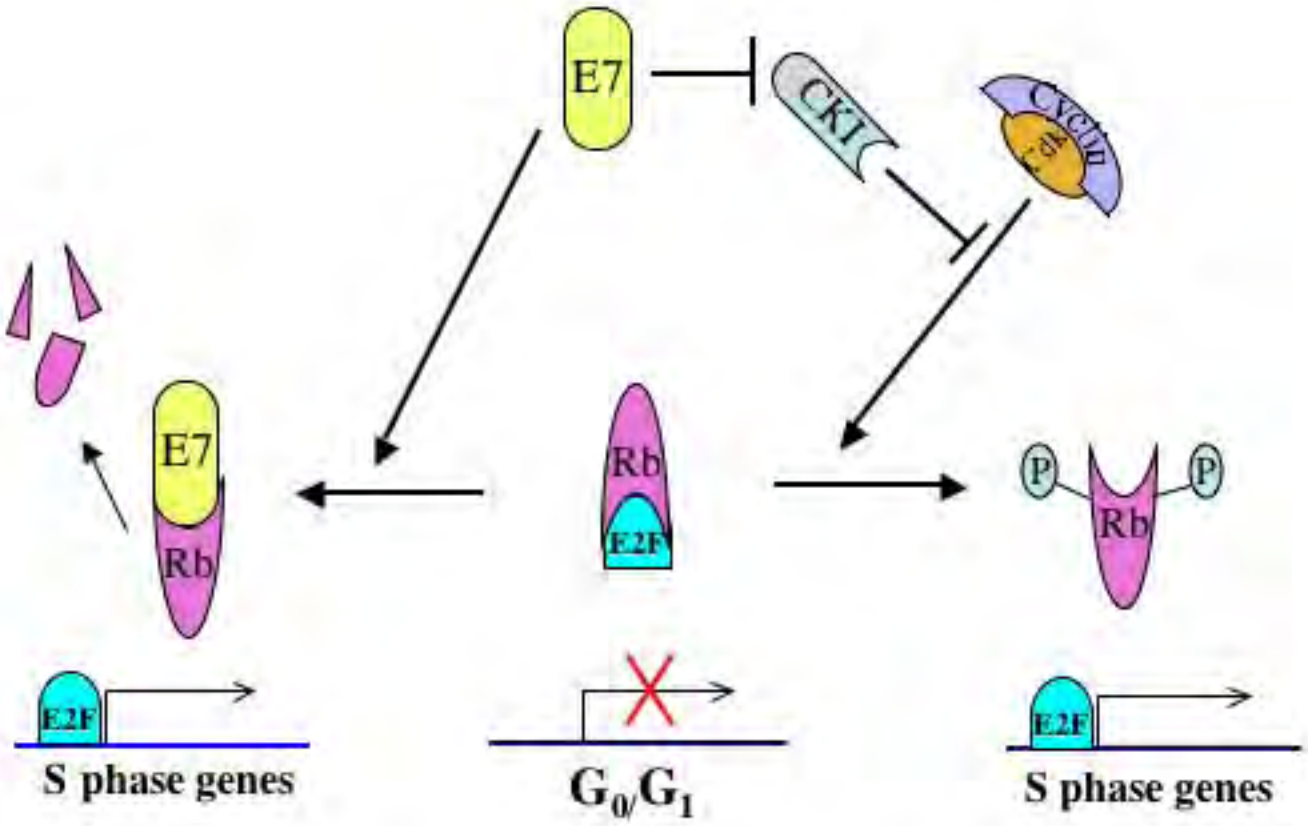
The E6 protein is approximately 150 amino acids and contains two zinc-binding domains. The oncogenic activities of E6 have been demonstrated in multiple biological assays, including immortalization of primary cells through activation of telomerase, transformation of established mouse fibroblasts, resistance to terminal differentiation of human keratinocytes, tumorigenesis in animals, modulation of apoptosis, and abrogation of cell cycle checkpoints (reviewed in (Fan & Chen, 2004; Mantovani & Banks, 2001; Rapp & Chen, 1998). The most prominent activity associated with E6 is its ability to bind and cause degradation of the p53 tumor suppressor. E6 first binds to the ubiquitin ligase E6-associated protein (E6-AP), and together they bind p53 as a complex (Huibregtse et al, 1993). E6-AP is then able to ubiquitinate p53, which marks p53 for proteolysis by the proteasome (Scheffner et al, 1993). High-risk HPV E6 proteins are also able to activate telomerase (Klingelhutz et al, 1996). These two functions of E6 are thought to be the major mechanisms through which E6 induces cellular transformation (Scheffner & Whitaker, 2003). Low-risk E6 proteins are only able to bind p53 and cannot target it for degradation. E6 also has functions independent of inactivating p53, including binding with and causing degradation of additional cellular proteins such as the proapoptotic protein Bak (Jackson et al, 2000), the DNA repair protein MGMT (Srivenugopal & Ali-Osman, 2002), and the transcription factor Myc (Gross-Mesilaty et al, 1998).

E7 is a nuclear protein of approximately 100 amino acids. Its amino terminus contains sequence homology to conserved region 1 (CR1) and CR2 of adenovirus E1A and SV40 (Barbosa et al, 1990; Phelps et al, 1988). The carboxy terminus contains a zinc-binding domain where E7 can dimerize/multimerize (Clemens et al, 1995). While E7 alone is able to immortalize human keratinocytes, the additional expression of E6 greatly enhances the frequency at which this occurs (Bedell et al, 1989; Halbert et al, 1991).

The most reported activity of E7 is its association with members of the retinoblastoma (Rb) tumor suppressor family, including p110Rb (Rb), p107, and p130 (Figure 1.7) (Davies et al, 1993; Dyson et al, 1989). Rb is one of the key regulators of S-phase progression through its interaction with the E2F family of transcription factors. Normally, Rb is considered active when hypophosphorylated and is then able to bind E2F transcription factors and thereby repress transcription of genes containing E2F promoter sites. Most of these genes are either required for the G1 to S phase transition or for S-phase replication and are therefore transcribed in a cell cycle dependent manner. (La Thangue, 1994; Slansky & Farnham, 1996) When Rb is hyperphosphorylated, it is unable to bind to E2F and the cell progresses through S-phase. E7 is able to bind Rb in its hypophosphorylated state (Dyson et al, 1989), which blocks binding to E2F, again promoting cell cycle progression through S-phase (Chellappan et al, 1992). The E7 protein from high and low risk types of HPV are able to bind to Rb, however, once bound to Rb, high-risk HPV-E7s are able to induce ubiquitin-mediated degradation of Rb (Giarre et al, 2001).

Figure 1.7. E7 expression results in down-regulation of Rb. When Rb is hyperphosphorylated by cdk/cyclinD complex, it is unable to bind to E2F and the cell progresses through S-phase. E7 is able to bind to Rb in its hypophosphorylated state, which blocks binding to E2F, again promoting cell cycle progression through S-phase. E7 is also able to inhibit function of the CKIs p21 and p27, which allows for phosphorylation of Rb.

Figure 1.7


E7
CKI
Cyclin
E7
Rb
Rb
E2F
P
P
Rb
E2F
E2F
S phase genes
G_0/G_1
S phase genes

While Rb may be E7's most common and most studied interacting partner, E7 has other interactions as well. Many of these interactions induce cell cycle progression and include: abrogating the inhibitory functions of cyclin-dependent kinase inhibitors p21 (Funk et al, 1997; Jones et al, 1997) and p27 (Zerfass-Thome et al, 1996); increasing the protein levels of the tyrosine phosphatase cdc25A (Katich et al, 2001); activating E2F2 transcription through binding to class I histone deacetylases (HDACs) (Longworth et al, 2005); degrading Rb through binding of the S4 subunit of the 26S proteasome (Berezutskaya & Bagchi, 1997).

Expression of both high-risk HPV E6 and E7 proteins is sufficient to immortalize primary human epithelial cells, however they are unable to induce transformation (Fan et al, 2005). Instead, it is proposed that the centrosome abnormalities, aberrant spindle pole formation, and genomic instability caused by E6 and E7 result in cells accumulating additional genomic aberrations necessary for malignant transformation. (Duensing et al, 2000; Duensing & Munger, 2002b). The sustained expression of E6 and E7 also appears to be essential for the maintenance of the transformed state of HPV-positive cells (Alvarez-Salas et al, 1998). Low-risk HPV types are unable to immortalize primary cells (Thomas et al, 2001).

Initially, integration of the viral genome, specifically in the E2 coding region, into the host genome was thought to be necessary for transformation, because of E2's ability to regulate E6 and E7 (Schwarz et al, 1985; Stoler et al, 1992). However, it has now been shown in studies of cervical cancer patients that approximately one-third of patients with malignant cervical lesions maintain an episomal HPV genome (Arias-Pulido et al, 2006; Cheung et al, 2006). Real-time PCR analysis showed that the amount of E7 in cervical

lesions is greatly increased compared to normal cells, although when normalized to viral content the number was not significant (Cheung et al, 2006); there was more E7 because there was more virus being produced. It has also been shown that E2 often acquires deletions that may inhibit its function (Arias-Pulido et al, 2006). All of these results suggest that E2 downregulation through integration or otherwise may not be necessary for increased E7 expression. Therefore, integration of the HPV genome may not be necessary for transformation.

In most cancer cells, potential initial causes of genomic instability are DNA damage and telomere shortening, both of which are found to occur in E6 and E7 expressing cells (Duensing & Munger, 2002b). Expression of HPV-16 E6 and/or E7 is associated with both structural and numerical chromosomal alterations (Coursen et al, 1997; Reznikoff et al, 1994; White et al, 1994). In normal human keratinocytes, expression of HPV-16 E7 rapidly induces abnormal centrosome duplication (within 48 h), whereas E6 has no immediate effect and takes longer to show centrosome abnormalities (Duensing et al, 2000). These HPV-16 E7-induced centrosome abnormalities represent an early event during neoplastic progression (Duensing et al, 2001). The ability of E7 to induce abnormal centrosome duplication is independent from its ability to degrade Rb or other pocket proteins (Duensing & Munger, 2003b). However, disregulation of cdk2 may contribute to increased centrosome duplication (Duensing et al, 2000), although lack of cdk2 does not seem to impede duplication (Tetsu & McCormick, 2003). Experiments involving both genetic and pharmacological cell cycle inhibitors suggest that while E7 may play a direct role in centrosome duplication,

the ability of E7 to induce centrosome duplication errors is connected to its ability to deregulate the cell cycle (Duensing & Munger, 2002a).

HPV-16 E7 and E6 separately expressed in mice under the control of an estrogen sensitive cervical cell specific promoter (K14) cause similar amounts of centrosome duplication/abnormalities. However, only those mice expressing E7 developed tumors, whereas the E6-expressing mice did not (Riley et al, 2003). Because the E6 and the E7 mice had similar amounts of centrosome duplication, but only the E7 mice developed tumors, this suggests that centrosome abnormalities alone may not be sufficient for transformation and that E7 has other cellular effects that lead to tumorigenesis.

Telomere shortening could lead to both structural and chromosomal instability (Gisselsson et al, 2002) and occurs in precursor lesions during cervical carcinogenesis (Zhang et al, 2004). It was also shown that telomere shortening was the primary cause of anaphase bridging as well as lagging chromosomes in colorectal cancer cells (Stewenius et al, 2005). Anaphase bridging is known to trigger complex rearrangements of chromosome structure (Stewenius et al, 2005), and is a phenomenon also observed in cervical intraepithelial neoplasia and cervical carcinoma (Therman et al, 1984). In E6 or E7 expressing primary human keratinocytes (PHKs), anaphase bridging correlates with metaphase lagging and structural chromosomal rearrangements (Duensing & Munger, 2002b). Expression of HPV-16 E7 alone was also associated with an increased number of cells exhibiting nuclear foci of phosphorylated histone H2AX as well as activation of cell cycle checkpoints triggered by DNA repair (Duensing & Munger, 2002b).

It has repeatedly been shown that expression of HPV-16 E6 and/or E7 induces polyploidy in both primary human fibroblasts and foreskin keratinocytes upon

microtubule disruption (Di Leonardo et al, 1997; Filatov et al, 1998; Khan & Wahl, 1998; Patel et al, 2004; Thomas & Laimins, 1998). However, E6 induces polyploidy more efficiently in the presence of E7 or in the context of HPV genome (Thomas & Laimins, 1998). Previous studies have explored the cellular mechanism through which this polyploidy was formed in HPV-16 E6 expressing cells (Liu et al, 2007).

1.3.3 Polyploidy and aneuploidy. Few women infected with a high-risk HPV type develop cervical cancer, and those that do usually have a long latency. Both of these observations suggest that presence of the HPV genome alone, specifically E6 and E7, is not sufficient for carcinogenesis. Rather, the CIN caused by these two oncogenes predisposes accumulation of additional genetic changes leading to transformation.

As previously mentioned, an important hallmark of human cancers, specifically those of epithelial origin, is CIN (Klausner, 2002). The most common manifestation of CIN is aneuploidy, the state of having an abnormal number of chromosomes (Edwards & Munger, 2004; Nasmyth, 2002). This is common in cancers of epithelial origin (Klausner, 2002), such as cervical cancers, where it can appear at early stages of cervical neoplasia (Duensing & Munger, 2003a; Heselmeyer et al, 1996). Several studies have also demonstrated that aneuploidy can originate from a polyploidy intermediate (Meraldi et al, 2002; Reid et al, 1996), and may therefore be an early event in multi-step carcinogenesis (Andreassen et al, 2001; Galipeau et al, 1996; Meraldi et al, 2002).

The cause of CIN is still unclear, although possibilities include mutations that deregulate telomere maintenance or mitotic integrity. However, such mutations have not yet been identified in spontaneous cancers (Duelli et al, 2007). It is possible that CIN

could be caused by a transient event, such as polyploidy or aneuploidy, that destabilizes the genome but does not permanently affect processes like mitosis or proliferation.

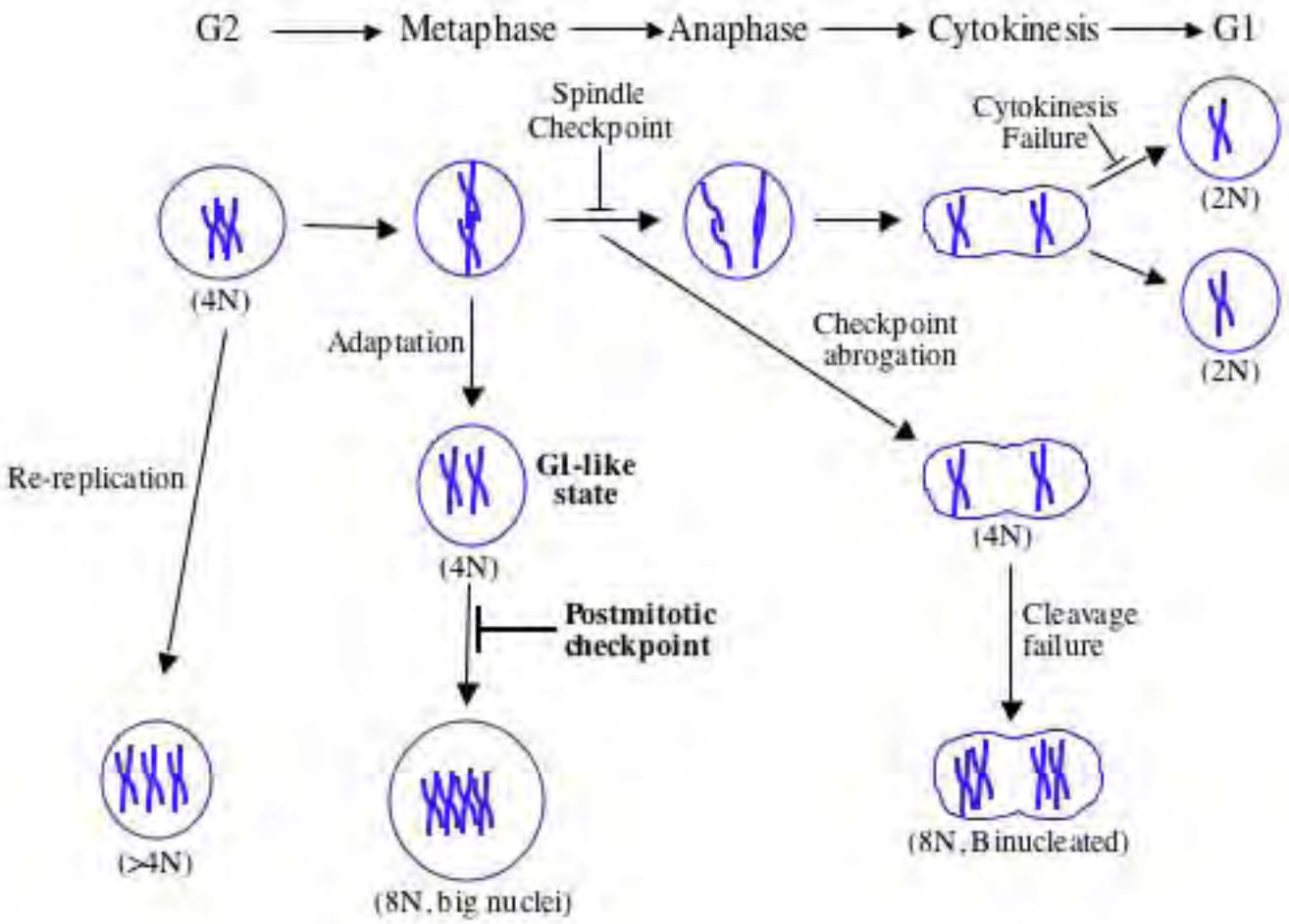
HPV-immortalized cell lines frequently acquire numerical chromosome changes (McCance et al, 1988; Oda et al, 1996; Pei et al, 1994; Solinas-Toldo et al, 1997). The HPV oncogenes E6 and E7, which are not sufficient for transformation (Fan & Chen, 2004), render normal human cells, including epithelial stem cells, genomically unstable, which predisposes them to accumulate additional chromosomal alterations necessary for malignant transformation (Duensing et al, 2000; Duensing & Munger, 2002b; White et al, 1994). The molecular basis leading to this genomic instability in HPV positive cells is not fully understood. In cervical tissue, polyploidy has recently been shown to not only occur as an early event in cervical carcinogenesis, but to predispose the cells to aneuploidy (Olaharski et al, 2006).

Polyploidization has also been shown to be a major cause of amplification of centrosomes (Meraldi et al, 2002), each of which may act as a mitotic spindle pole (Quintyne et al, 2005), preventing the cells from segregating properly. If these cells then complete cytokinesis, the resulting cells will most likely be aneuploid (Edwards & Munger, 2004). Other recent studies have shown that tetraploid, but not diploid, mouse or human cells induce tumor formation in mice (Duelli et al, 2007; Fujiwara et al, 2005). These studies highlight the potential importance of polyploidy in carcinogenesis.

1.3.4 Mechanisms to Polyploidy. There are several major possibilities through which polyploidy can be induced (Figure 1.8). Most of these mechanisms are involved in improper execution of the cell cycle, including checkpoints that regulate whether the

Figure 1.8. Mechanisms to polyploidization. There are several conflicting mechanisms through which polyploidy can be induced in E7 expressing cells. These include abrogation of the spindle assembly checkpoint followed by cleavage failure, cleavage failure alone, re-replication, or adaptation to the spindle checkpoint followed by post-mitotic checkpoint abrogation.

Figure 1.8


G2
Metaphase
Anaphase
Cytokinesis
G1
Spindle Checkpoint
Cytokinesis Failure
(2N)
(4N)
Adaptation
Checkpoint abrogation
(2N)
Re-replication
G1-like state
(4N)
(4N)
Postmitotic checkpoint
Cleavage failure
(>4N)
(8N, big nuclei)
(8N, Binucleated)

genome is properly replicated, repaired, and segregated. It has previously been shown that defects in checkpoint functions may contribute to genomic and chromosomal instability [as reviewed in (Hartwell & Kastan, 1994; Paulovich et al, 1997), specifically polyploidization. There are two checkpoints in eukaryotic cells where defects may lead to cells acquiring polyploid genomes, including the spindle assembly checkpoint and the more recently described postmitotic checkpoint (Lanni & Jacks, 1998).

Once in mitosis, the mitotic spindle assembly checkpoint controls the division of the chromosomes. Specifically, proper attachment of the microtubules to the kinetochores and proper alignment of the chromosomes at the metaphase plate are monitored by the spindle assembly checkpoint. Activation of the checkpoint delays chromosome segregation until the mitotic spindle apparatus is correctly assembled (Musacchio & Hardwick, 2002). The spindle assembly checkpoint is monitored by the interplay between two ubiquitin-protein ligases, the anaphase promoting complex/cyclosome (APC/C) and the Skp1-cullin-F-box (SCF) protein (Vodermaier, 2004). Although an early study with fibroblasts from $p53^{-/-}$ mouse embryos suggested that p53 might play a role in the spindle assembly checkpoint (Cross et al, 1995), subsequent studies demonstrated that p53 is not a key regulator of this checkpoint control mechanism (Lanni & Jacks, 1998).

The spindle checkpoint is activated until the chromosomes are properly attached to the spindle and aligned at the metaphase plate. Cells that do not complete mitosis often suffer cleavage failure as well. Therefore, if the spindle checkpoint becomes abrogated, but the cells still fail to cleave, then the cells will have a 4N DNA content. These cells may not trigger the post-mitotic checkpoint, and therefore progress through

the cell cycle becoming polyploid. Cleavage failure may also occur due to disruption of the microtubule architecture during cytokinesis and abscission. Once cell division fails, another round of DNA replication may ensue, which could also result in polyploidy. Although mutations in spindle assembly checkpoint genes could cause aneuploidy and predispose cells to cancer (Hanks et al, 2004), loss-of-function mutations in these genes are rarely observed in human cancers (Hernando et al, 2001).

Cells with intact spindle assembly checkpoint activity that become arrested in metaphase for prolonged periods of time eventually adapt to the checkpoint and progress into a G1-like state with 4C DNA content (Lanni & Jacks, 1998; Margolis et al, 2003). A proposed p53- and pRb-dependent postmitotic checkpoint then prevents the cells from continuing through G1 and S (Khan & Wahl, 1998; Lanni & Jacks, 1998). Although previously referred to as a "tetraploidy" checkpoint (Margolis et al, 2003), it appears that the structural integrity and dynamics of the microtubules, rather than tetraploidy per se, triggers cell cycle arrest at this checkpoint (Uetake & Sluder, 2004; Wong & Stearns, 2005).

The postmitotic checkpoint shares many features with the G1 checkpoint, including high concentrations of p21 and hypophosphorylated Rb during cell cycle arrest (Borel et al, 2002; Meraldi et al, 2002). While p53 appears to play a key role in mediating the postmitotic checkpoint (Andreassen et al, 2001; Di Leonardo et al, 1997; Lanni & Jacks, 1998; Sablina et al, 1999) and p21 is responsible for at least part of this p53-mediated postmitotic arrest response (Khan & Wahl, 1998; Lanni & Jacks, 1998; Stewart et al, 1999), p53-independent, Rb-dependent activities cannot be ruled out.

Another mechanism that can lead to polyploidization is re-replication, a process of multiple rounds of DNA replication without an intervening mitosis. Cells complete S-phase and enter G2, but then proceed directly into S-phase again. Re-replication is an inefficient process to replicate DNA and often yields incomplete copies of the genome resulting in polyploidy or aneuploidy.

There are many conflicting hypotheses concerning how expression of E7 leads to polyploidization. Chapter III explores the different mechanisms through which expression of E7 and subsequent Rb-downregulation leads to polyploidization. Once the specific mechanism is ascertained, therapies may be developed to prevent accumulation of polyploidy in E7 expressing and/or Rb-downregulated, untransformed cells. This may subsequently prevent the accumulation of numerical genomic aberrations that could cooperate with E7 and lead to carcinogenesis.

Chapters II and III of this dissertation present two examples of the multi-step nature of cancer. While certain genetic mutations or cellular changes may not lead directly to cancer, such as expression of E7 or the presence of *Cbfb/MYH11*, they may increase the likelihood of acquiring mutations that do cause transformation. First, I explore how the lack of *Cbfb* affects *Cbfb/MYH11* positive cells. The presence of the fusion protein does not lead to transformation on its own, and *Cbfb* will either act as a cooperating mutation or will predispose the cell to acquiring other Class I mutations for leukemogenesis. One known *Cbfb/MYH11* cooperating Class I mutation is the HPV oncogene E7, which causes acceleration of the progression from G1 to S by degrading pRb (Yang et al, 2002). On the other hand, in HPV positive cells, E7 alone is not

sufficient for transformation. However, E7 does lead to polyploidy, which in turn can lead to aneuploidy and CIN, and the additional mutations accumulated from CIN may lead to transformation. Therefore, in Chapter III of this dissertation I determine the mechanism that leads to polyploidy in Rb-downregulated E7 cells.

CHAPTER II

CBFβ MODULATES CBFβ-SMMHC-ASSOCIATED ACUTE MYELOID LEUKEMIA IN MICE

Preface

All of the experiments in this chapter were done by the author with the exception of the human AML sample analysis, which was done by Peter Valk, Department of Hematology, Erasmus University Medical Center, Rotterdam, Netherlands. The work in this section has been published in:

Heilman SA, Kuo YH, Goudswaard CC, Valk PJM, Castilla LH. The role of *Cbfb* in *Cbfb/MYH11* associated leukemogenesis. *Cancer Research*. 2006. 66:11214-8.

Abstract

The gene encoding the core-binding factor beta (CBFβ) is altered in acute myeloid leukemia (AML) by an inversion in chromosome 16, resulting in expression of the fusion protein CBFβ-SMMHC. Previous studies have determined that this oncoprotein interferes with hematopoietic differentiation and proliferation, and participates in leukemia development. In this study, evidence is provided that the remaining wild-type Cbfβ modulates the oncogenic function of this fusion protein. Bone marrow progenitor cells from *Cbfb-MYH11* knock-in and *Cbfb*-knockout mice are used to study the effect of Cbfβ in hematopoietic differentiation and leukemia progression. Cbfβ was determined to play an important role in proliferation of hematopoietic progenitors expressing Cbfβ-SMMHC *in vitro*. In addition, Cbfβ-SMMHC-mediated leukemia development is accelerated in the absence of a wild-type copy of Cbfβ. These results provide evidence that the ratio of expression levels between Cbfβ and Cbfβ-SMMHC directly affects the pathogenesis of leukemia and indicate that CBF-specific therapeutic molecules should target CBFβ-SMMHC function while maintaining CBFβ activity.

Introduction

The CBF transcription factor is the most common target of chromosomal rearrangements in human AML, including the fusion genes *CBFB-MYH11* and *RUNX1-ETO* (Figure 1.1), as well as point mutations in *RUNX1* (Reilly, 2005). CBF is a heterodimeric transcription factor that consists of a DNA binding α-subunit, encoded by one of three members of the RUNX family (*RUNX1, RUNX2*, and *RUNX3*), and a β-subunit encoded by the *CBFB* gene that confers increased DNA-binding affinity to the complex. Mouse studies have shown that in hematopoiesis, the CBF heterodimer Cbfβ:Runx1 regulates expression of genes with critical functions in hematopoietic differentiation of lymphoid and myeloid lineages. Studies in mouse have determined that $Cbfb^{-/-}$ and $Runx1^{-/-}$ embryos fail to develop embryonic definitive hematopoiesis and die at midgestation (Okuda et al, 1996; Sasaki et al, 1996; Wang et al, 1996a; Wang et al, 1996b). This phenotype was rescued in $Cbfb^{-/-}$ mice by expressing *Cbfb* from the hematopoietic specific promoters *Tie2* or *GATA1*, further underscoring the key role of Cbfβ during hematopoietic differentiation (Miller et al, 2002; Yoshida et al, 2002).

Approximately 12% of AML patients present a chromosome 16 inversion, inv(16), that breaks and joins the first 5 exons of *CBFB* with the second half of the smooth muscle myosin heavy chain gene *MYH11* (Look, 1997). The resulting fusion gene, *CBFB-MYH11,* encodes the CBFβ-SMMHC fusion protein, which retains the Runx-binding domain from Cbfβ and the multimerization capacity of the myosin sequence. Studies in the mouse have shown that Cbfβ-SMMHC is a dominant inhibitor of CBF function, as $Cbfb^{+/MYH11}$ heterozygous knock-in embryos expressing the fusion protein also failed to develop definitive hematopoiesis (Castilla et al, 1996). Induction of

Cbfβ-SMMHC expression or Runx1-loss in adult bone marrow (BM) does not seem to affect the maintenance of long-term hematopoietic stem cells (HSC) (Growney et al, 2005; Ichikawa et al, 2004; Kuo et al, 2006). However, Cbfβ-SMMHC expression, acting as a Class II mutation, reduces HSC function by inhibiting multilineage repopulation and by creating a myeloid progenitor predisposed to leukemia development.

It has been suggested that Cbfβ-SMMHC may exert an incomplete block of CBF function. In this study, the hypothesis that Cbfβ is able to modulate the effect of Cbfβ-SMMHC in adult hematopoiesis and leukemogenesis is tested. Mice with a *Cbfb* knock-out allele and a conditional *Cbfb-MYH11* knock-in allele were used to study adult myeloid differentiation and leukemia progression. Colony-forming assays revealed that Cbfβ plays a critical role in proliferation of myeloid progenitors. In addition, loss of Cbfβ in bone marrow cells expressing Cbfβ-SMMHC induces AML development in the mouse with faster onset than controls. These results provide evidence that Cbfβ modulates hematopoietic differentiation and Cbfβ-SMMHC-mediated leukemia development.

Materials and Methods

Generation of triple *Mx1Cre;Cbfb*$^{-/MYH11}$ transgenic mice. The design of the conditional *Cbfb*$^{+/MYH11}$ knock-in mice has been previously described (Kuo et al, 2006). Expression of CBFβ-SMMHC was induced in 3-weeks old mice by activation of Cre recombinase from the *Mx1Cre* transgene using 1 to 3 doses of polyinosinic-polycytidylic acid (pIpC) every other day (Kuhn et al, 1995). Heterozygous *Cbfb*$^{-/+}$ knock-out mice were generously provided by Nancy Speck (Wang et al, 1996b). For this study, all mice were maintained in the 129SvEv strain. In the transplantation assays, $1x10^6$ leukemic cells were transplanted into sublethally irradiated syngenic recipients as described previously (Landrette et al, 2005).

Mice were genotyped with DNA from tail snips obtained at weaning (3 weeks of age). Genotyping was done by PCR with the following primers:

Cre → CreA forward CCGGGCTGCCACGACCAA
CreA reverse GGCGCGGCAACACCATTTTT
Cbfb knock-in allele → Hyg forward CCATCGTCGAGATCCAGACATG
Hyg reverse GTATATGCTCCGCATTGGTCTTG
Cbfb null allele → pgk5' forward CTGACTGCGTTAGCAATTTAAC
Cbfb3' reverse GAAGACCAATAAATGAGAATAAATGC
wild-type *Cbfb* → I-4 forward ATAAGCAGCAAATAGGTAGAGTG
I-54 reverse CCAGCAGCTGTGAAACTC

Cells isolated from peripheral blood, bone marrow, or methylcellulose colonies were harvested. The red blood cells in the peripheral blood and bone marrow were lysed with Puregene Red Blood Cell (RBC) lysis solution (Gentra Systems). The remaining white blood cells were then incubated in proteinase K for 1 hour at 55°C. PCR for the deletion was done with the following primers (Figure 2.1):

4Lx1 forward CAGTGCTCTTGCTAGTGGATC
kT3.1 reverse for the deletion GCTCAACAGTATCAAGAGTCG
h5/6 reverse for the undeleted CTTCTTGCCTCCATTTCCTCC

Immunoblotting. Western blot was performed as previously described (Kuo et al, 2006). Briefly, total protein extracts were separated by SDS-PAGE and transferred to a PVDF membrane. Membranes were blotted with antibodies against Cbfβ (β-141, kindly provided by Nancy Speck, Dartmouth Medical School) and α-tubulin (Sigma, St. Louis, MO).

Histopathology. Peripheral blood was obtained from the retroorbital sinus of the mouse. White blood cell counts were determined with a hemocytometer after lysing the red blood cells with RBC lysis solution, washing with PBS, and staining with Trypan Blue (Gibco-Invitrogen) in a 1:1 volume. Cellular morphologies were identified after staining peripheral blood smears with Wright-Giemsa stain. Histology analysis was performed on sections from paraffin embedded blocks and stained with Hematoxilin and Eosin (Histoserv, Gathesburg MD). Pictures were taken using a Zeiss Axioskop 40 microscope using 10x, 40x, and 100x lenses and a Zeiss AxioCam Mrc camera.

Flow cytometry Analysis. Cell-surface antigens were detected by standard immuno-fluorescence assays using phycoerythrin (PE)-conjugated monoclonal antibodies to Mac1 (1:800), Gr1 (1:800), CD3 (1:400), B220 (1:800), and Sca1 (1:400), Fluorescein (FITC)-conjugated monoclonal antibodies to Mac1, Gr1, CD3, and B220, Allophycocyanin (APC)-conjugated monoclonal antibody to c-kit/CD117 (1:1600), PE-Cy7-conjugated

monoclonal antibody to FcγR (1:50), and APC-Cy7-conjugated monoclonal antibody to c-kit (1:100). PerCP-Cy5.5-conjugated streptavidin (1:200) was used to detect the biotinylated antibody to CD34 (1:50). Appropriate isotype controls were included in all experiments. All antibodies were obtained from Becton-Dickinson Biosciences (San Jose, CA) except for APC-Cy7 and Pe-Cy7, which came from eBiosciences (San Diego, CA). Fluorescence was analyzed on an LSRII and data was analyzed using FloJo 6.1.1.

Colony forming assays. Mice with genotypes *Cbfb*$^{+/56M}$, *Cbfb*$^{+/MYH11}$, *Cbfb*$^{-/56M}$, and *Cbfb*$^{-/MYH11}$ were injected with pIpC at weaning, every other day. Two days after the second injection, BM cells were harvested, and $1x10^4$ white blood cells were plated in duplicate in methylcellulose supplemented with cytokines IL-3, IL-6 and SCF and erythropoietin (Methocult-3434, Stem Cell Technologies, Vancouver) in 35 mm non-tissue culture treated dishes. The number of erythroid and total colonies was scored at day 4. The number of myeloid colonies was scored at day 7. Single colonies were harvested and either cytospun for cytology analysis or placed into lysis buffer for PCR analysis. Pictures were taken with a Zeiss AxioCam Mrc camera.

Statistical considerations. Differences in survival functions between groups were evaluated by Kaplan-Meier product limit survival analysis using the Tarone-Ware test to test the hypothesis of overall equivalence. In the presence of significant overall differences pair-wise comparisons were made between the non-control groups using Tarone-Ware tests with a Sidak adjustment to compensate for the additive Type I error due to multiple comparisons.

Analysis of human AML samples. Patients had a diagnosis of primary AML, confirmed by cytological examination of blood and bone marrow. After informed consent, bone marrow aspirates or peripheral blood samples were taken at diagnosis (n=285) and processed for the Affymetrix U133A GeneChip analysis (Valk et al, 2004). For PCR and sequence analyses, cDNA prepared from 50ng of RNA was used for all PCR amplifications. The *CBFB* coding region was sequenced for 27 inv(16)$^{+}$ AML samples by cDNA amplification using the primers:

CBFB-FOR 5'-CAGAGAAGCAAGTTCGAGAACG-3' with

CBFB-REV 5'-GTTTGAGGTCATCACCACCAC-3'

CBFB-FOR with *CBFB6* 5'-GTCTTGTTGTCTTCTTGCCAG-3'

with the recipe 25mM dNTP, 15 pmol primers, 2mM $MgCl_2$, Taq polymerase and 10xbuffer (Invitrogen Life Technologies, Breda, Netherlands). Cycling conditions for both primer sets consisted of a denaturing cycle for 5 min at 94˚C followed by 30 cycles for amplification (1 min 94˚C, 1 min 62˚C, 1 min 72˚C), and a final extension cycle for 7 min at 72˚C. PCR products were purified using the Multiscreen-PCR 96-well system (Millipore, Bedford, MA) followed by direct sequencing with CBFB-FOR, CBFB-REV and CBFB6 using an ABI-PRISM3100 genetic analyzer (Applied Biosytems, Foster City, CA).

Results

Loss of Cbfβ reduces proliferation capacity of myeloid progenitors expressing Cbfβ-SMMHC *in vitro*. It has recently been shown that BM cells expressing Cbfβ-SMMHC accumulate abnormal myeloid progenitors able to form myeloid colonies *in vitro* (Kuo et al, 2006). To assess whether this effect is dependent on the presence of Cbfβ, colony-forming unit (CFU) assays were performed using BM cells from heterozygous floxed (*Cbfb*$^{+/56M}$), hemizygous floxed (*Cbfb*$^{-/56M}$), heterozygous restored (*Cbfb*$^{+/MYH11}$), and hemizygous restored (*Cbfb*$^{-/MYH11}$) mice (Figure 2.1). The expression of the fusion protein Cbfβ-SMMHC, which switches the floxed to the restored allele, was induced by pIpC treatment-mediated Cre activation using the *Mx1Cre* transgene. Excision of the knocked-in sequence was confirmed by PCR analysis.

The number of colony forming units (CFUs) at day 7 was significantly reduced in hemizygous restored BM progenitor cells expressing Cbfβ-SMMHC when compared to control groups (Figure 2.2A). In addition, the loss of one *Cbfb* allele did not affect CFU formation (heterozygous floxed vs. hemizygous floxed), indicating that the observed phenotype results from a reduction below 50% of the *Cbfb* transcript. The presence of Cre-lox mediated deletion was confirmed in CFUs from hemizygous restored plates by PCR analysis (Figure 2.2B). The same increases and decreases in colony number were seen for the blast forming erythroid unit (BFU-E) colonies counted on day 4 as well (Figure 2.2A). Interestingly, the size of the colonies was markedly reduced in hemizygous restored plates when compared to control plates (Figure 2.2C).

Cytology analysis of day-7 CFUs revealed the presence of all myeloid forms, with a small but consistent increase of blastlike immature cells in hemizygous restored

Figure 2.1. *Cbfb* alleles used in this study. Exons 1 to 6 of the *Cbfb* gene are shown in boxes, and the encoded protein is shown on the right. The *Cbfb* knock-out allele includes a Neomycin (N) gene fused to the 3'-end of exon 5. The floxed $Cbfb^{56M}$ allele includes exons 5 and 6 and a Hygromycin gene (H) between loxP sites, followed by exon 5 fused to the 3' *MYH11* sequence and a Neomycin gene. Upon Cre-mediated loxP deletion, Cbfβ-SMMHC is induced in the $Cbfb^{MYH11}$ restored allele. PCR analysis for loxP deletion was done using primers 4Lx1, KT3, and h5/6.

Figure 2.1

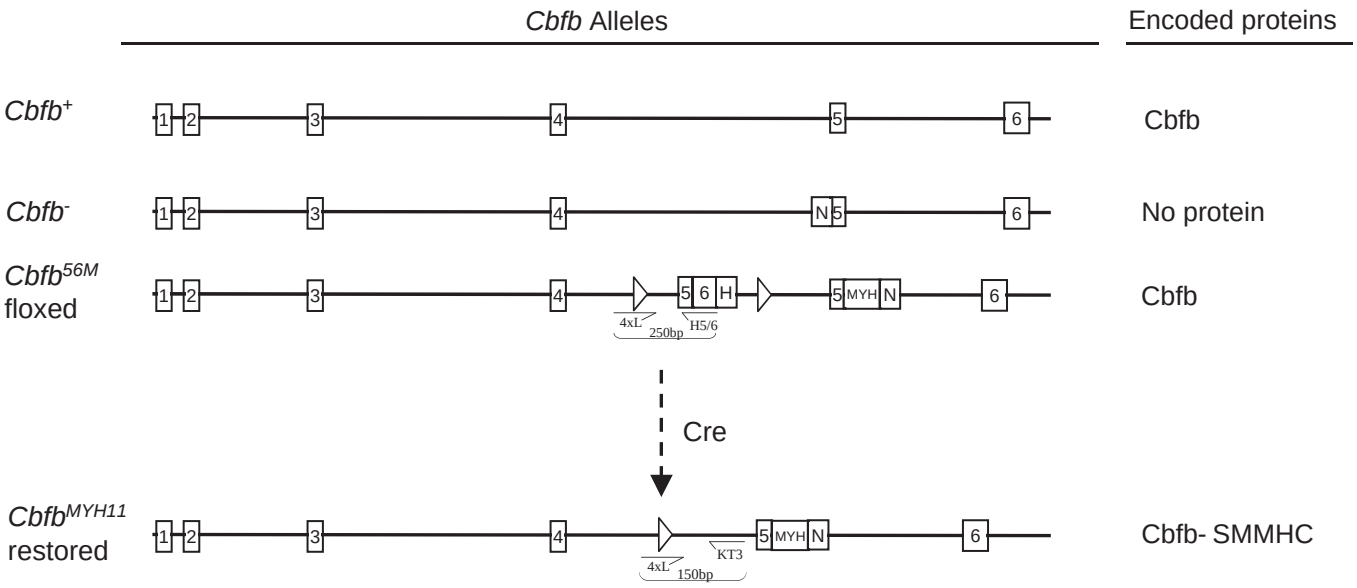
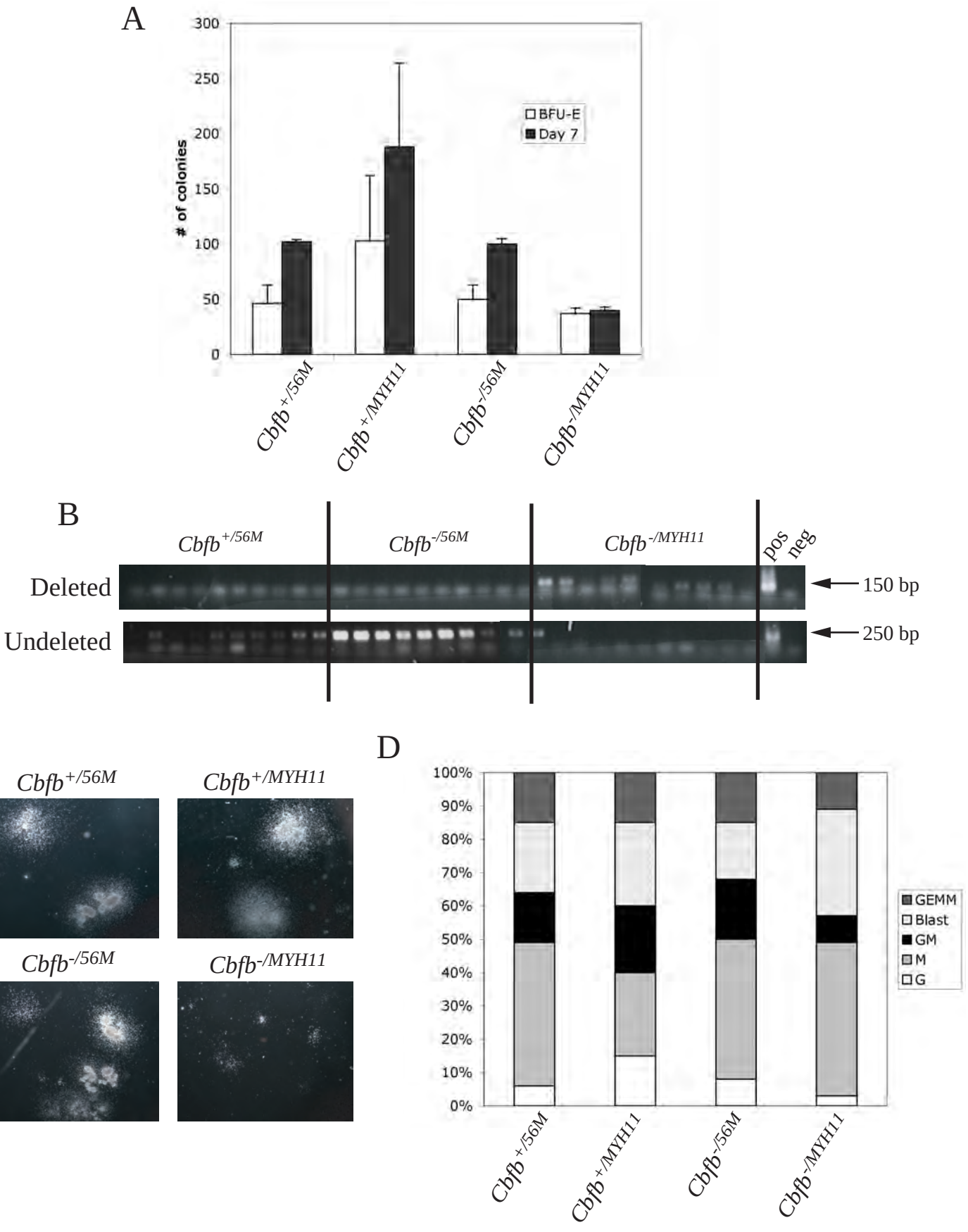

Cbfb Alleles
Encoded proteins
Cbfb+
1
2
3
4
5
6
Cbfb
Cbfb-
1
2
3
4
N
5
6
No protein
*Cbfb*56M
floxed
1
2
3
4
5
6
H
5
MYH
N
6
4xL
250bp
H5/6
Cbfb
Cre
*Cbfb*MYH11
restored
1
2
3
4
5
MYH
N
6
KT3
4xL
150bp
Cbfb- SMMHC

Figure 2.2. **Cbfβ modulates BM myeloid proliferation *in vitro*.** Colony forming assays in methylcellulose cultures using $1x10^4$ BM hematopoietic progenitor cells with heterozygous floxed (*Cbfb*$^{+/56M}$), heterozygous restored (*Cbfb*$^{+/MYH11}$), hemizygous floxed (*Cbfb*$^{-/56M}$) or hemizygous restored (*Cbfb*$^{-/MYH11}$) genotypes. **A.** Scored at days 4 and 7. Data is from three independent experiments, each in duplicate. **B:** PCR for the deletion of the conditional knock-in exons 5 and 6. **C**. Representative picture of colony size (amplification 50x) from day 7. **D**. Histogram representation of cytology analysis of day 7 GEMM-, Blast-, GM-, M-, and G-CFUs. Data is from three independent experiments, each in duplicate.

Figure 2.2


A
300
250
200
150
100
50
0
# of colonies
BFU-E
Day 7
Cbfb +/56M
Cbfb +/MYH11
Cbfb-/56M
Cbfb-/MYH11
B
Cbfb+/56M
Cbfb-/56M
Cbfb-/MYH11
pos
neg
Deleted
Undeleted
150 bp
250 bp
C
Cbfb+/56M
Cbfb+/MYH11
Cbfb-/56M
Cbfb-/MYH11
D
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
GEMM
Blast
GM
M
G
Cbfb +/56M
Cbfb +/MYH11
Cbfb-/56M
Cbfb-/MYH11

colonies (Figure 2.2D). However, the *Cbfb*$^{+/MYH11}$ cells formed more CFU-G (granulocyte) and CFU-GM (granulocyte/macrophage) colonies, and the *Cbfb*$^{-/MYH11}$ cells appeared to have an impairment in granulocyte formation (decrease in CFU-G) and had an increase in macrophage/monocyte (CFU-M) formation. The BFU-E colonies seen in all genotypes at day 4 and the variety of myeloid colonies (CFU-GM, CFU-GEMM (granulocyte, erythroid, macrophage, monocyte)) seen at day 7 distinguish these cells as having maintained both erythroid and myeloid differentiation potential in all of these genotypes. These results revealed that the loss of Cbfβ significantly reduces the proliferation capacity of BM myeloid progenitors expressing CBFβ-SMMHC *in vitro*. These results are in accordance with studies using *Cbfb*$^{-/-}$ embryonic stem cells, in which loss of Cbfβ was shown to inhibit formation of myeloid colonies (Miller et al, 2001). Furthermore, while ectopic Cbfβ expression restored colony formation in *Cbfb*$^{-/-}$ embryonic stem cells, CBFβ-SMMHC was intrinsically unable to restore CBF function (Miller et al, 2001).

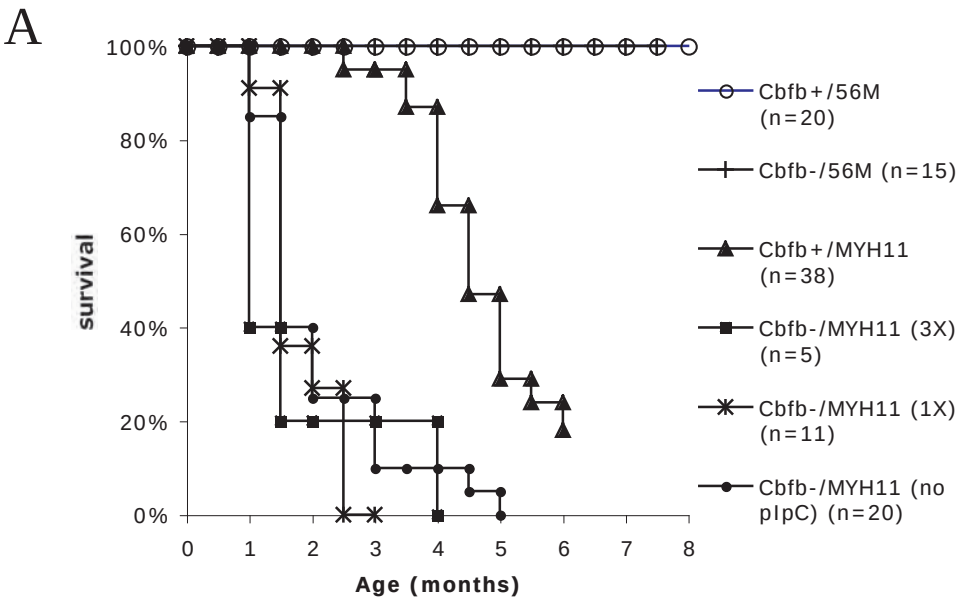
Hemizygous BM cells expressing Cbfβ-SMMHC show higher susceptibility to AML. The *Cbfb*$^{+/MYH11}$ mice expressing Cbfβ-SMMHC and Cbfβ succumb to AML with a median latency of 5 months due to acquisition of random cooperating Class I mutations (Kuo et al, 2006). This latency is dose-dependent as mice treated with 3 pIpC doses developed AML with a median latency of 5 months, mice treated with 1 pIpC dose with a median latency of 8 months, and untreated mice remained disease free up to one year (end of experiment).

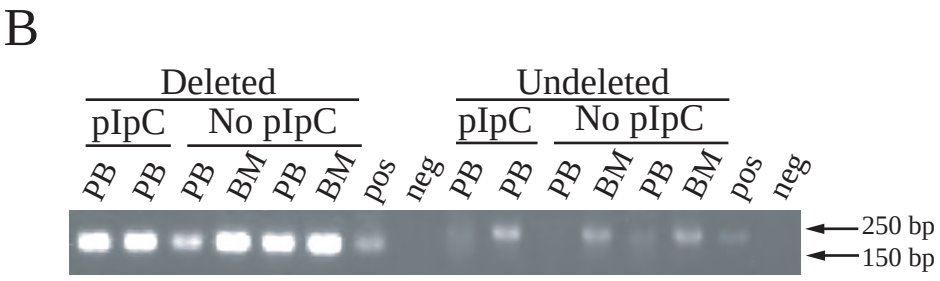
To test whether the presence of Cbfβ has an effect on Cbfβ-SMMHC-mediated AML, we compared heterozygous restored to hemizygous restored mice, after treatment with 3 doses of pIpC. In the absence of Cbfβ, 100% of mice with BM cells expressing Cbfβ-SMMHC developed AML with a significant acceleration of disease onset (median latency of 1.5 ± 0.5 months; $p<1e^{-5}$; Figure 2.3A). Surprisingly, untreated *Cbfb*$^{-/MYH11}$ -mice also developed AML with a similar latency to that of the induced group (Figure 2.3A). It has previously been reported that Cre expression from the *Mx1Cre* transgene is leaky in mice not treated with pIpC (Chan et al, 2004). In this study, all AML samples from treated and untreated groups exhibited deletion of the floxed sequence by PCR analysis (Figure 2.3B), and Cbfβ-SMMHC expression was detected by western blot analysis (Figure 2.3C). Furthermore, secondary transplantation of *Cbfb*$^{-/MYH11}$ AML cells derived from treated or untreated mice produced leukemia in sublethally irradiated recipients with a median latency of 6 weeks (data not shown). These results indicate that Cbfβ-SMMHC-induced AML development is accelerated in the absence of Cbfβ.

Expression of Cbfβ is critical for the emergence of embryonic HSCs (Miller et al, 2002). However, while 50% reduction of Cbfβ levels does not seem to alter hematopoiesis, the effect of Cbfβ levels below 50% in adult BM progenitors has not been previously tested. Based on our results, however, we cannot definitively rule-out that Cbfβ-loss could induce AML in the absence of Cbfβ-SMMHC. Future studies using conditional *Cbfb* knock-out mice or Cbfβ-shRNA approaches may directly address this point. However, considering that loss of Runx1 in HSCs using conditional knock-out mice does not induce AML (Growney et al, 2005; Ichikawa et al, 2004), the disease phenotype observed in our study is likely dependent on the presence of Cbfβ-SMMHC.

Figure 2.3. Loss of wild-type *Cbfb* accelerates Cbfb-SMMHC-mediated AML. A. Kaplan-Meier survival curve of mice expressing Cbfβ-SMMHC in the presence or absence of Cbfβ. $Cbfb^{+/56M}$ mice (open circles), $Cbfb^{-/56M}$ mice (tics), $Cbfb^{+/MYH11}$ mice with 3 shots of pIpC (triangles), $Cbfb^{-/MYH11}$ mice with 3 shots of pIpC (squares), $Cbfb^{-/MYH11}$ mice with 1 shot of pIpC (crosses), and $Cbfb^{-/MYH11}$ mice with no pIpC (closed circles). **B.** PCR analysis of peripheral blood and bone marrow samples for the deletion of the conditional knock-in of exons 5 and 6 in $Cbfb^{-/MYH11}$ mice that developed leukemia without induction with pIpC. **C.** Western blot analyses of Cbfβ-SMMHC and β-actin in AML samples derived from restored *Cbfb* mice treated (or untreated) with pIpC. The *Cbfb* genotype of the AML cells is shown on the top.

Figure 2.3


A
100%
80%
60%
40%
20%
0%
survival
0
1
2
3
4
5
6
7
8
Age (months)
Cbfb+/56M (n=20)
Cbfb-/56M (n=15)
Cbfb+/MYH11 (n=38)
Cbfb-/MYH11 (3X) (n=5)
Cbfb-/MYH11 (1X) (n=11)
Cbfb-/MYH11 (no pIpC) (n=20)


B
Deleted
Undeleted
pIpC
No pIpC
pIpC
No pIpC
PB
PB
PB
BM
PB
BM
pos
neg
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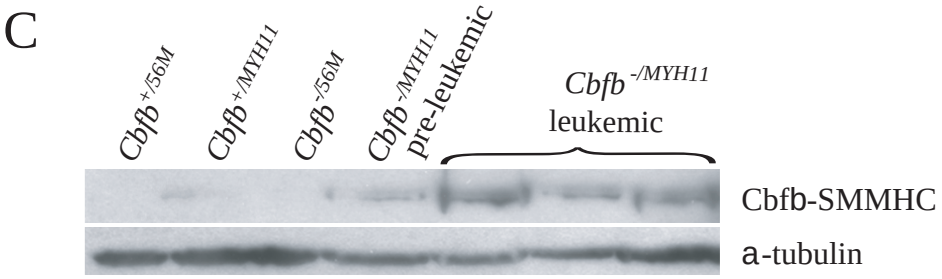
PB
PB
BM
PB
BM
pos
neg
250 bp
150 bp


C
Cbfb+/56M
Cbfb+/MYH11
Cbfb-/56M
Cbfb-/MYH11 pre-leukemic
Cbfb-/MYH11 leukemic
Cbfb-SMMHC
a-tubulin

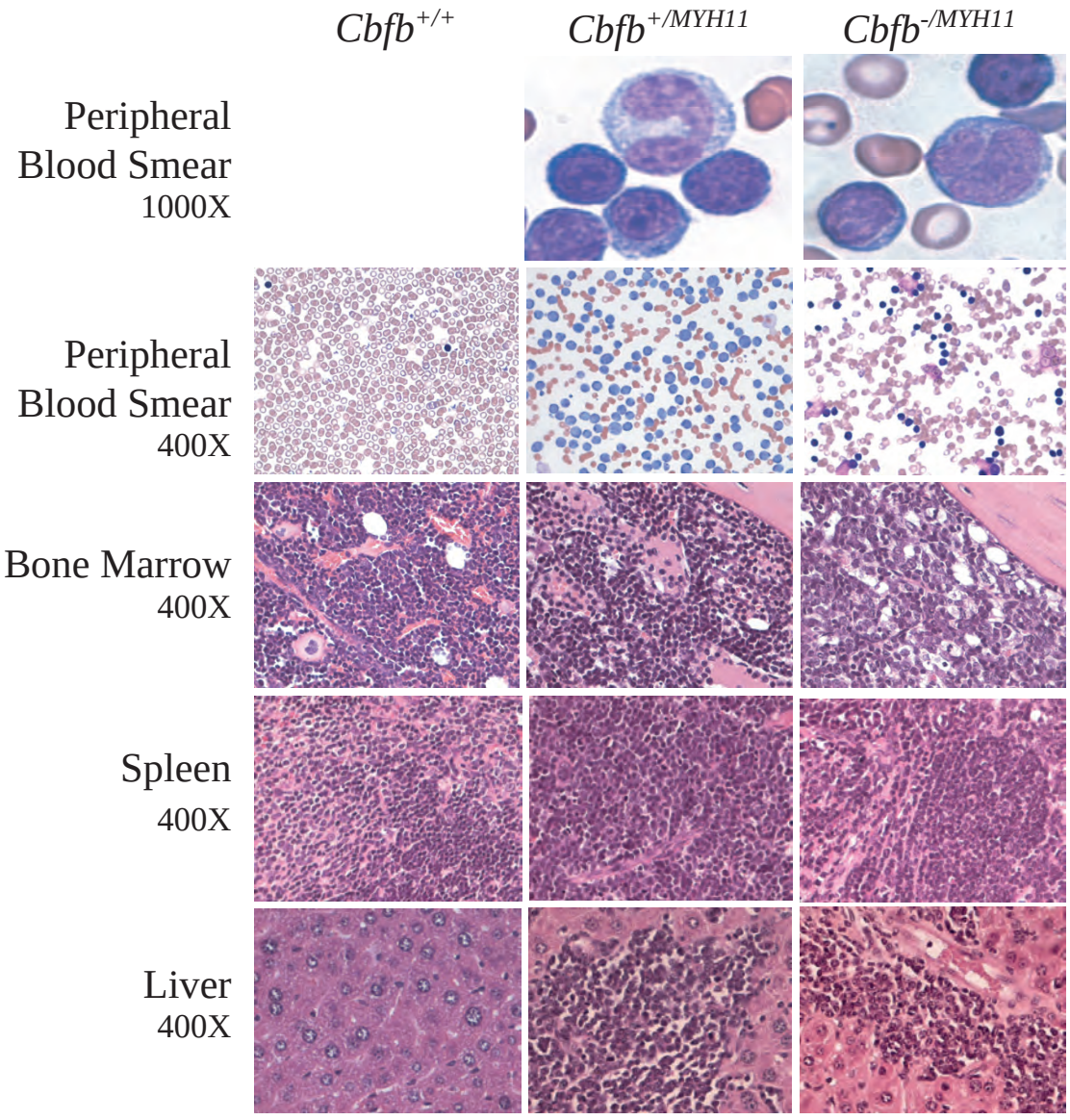
Importantly, our findings that uninduced *Cbfb*$^{-/MYH11}$ mice succumb with AML at short latency and that secondary AML develops in sublethally irradiated recipients, strongly support the model that the disease results from the clonal expansion of a few abnormal BM hematopoietic progenitors within a normal hematopoietic environment.

Disease pathology is dictated by Cbfβ-SMMHC. The hemizygous leukemic *Cbfb*$^{-/MYH11}$ mice presented an increase in white blood cell count (mean = 41.7 ± 74.7 x10^6/mL) when compared to healthy littermates (mean = 4 x10^6/mL), although lower than that observed in heterozygous leukemic *Cbfb*$^{+/MYH11}$ mice (mean = 197.5 ± 170.3 x10^6/mL). As previously seen in heterozygous restored leukemic mice, the leukemic cells from the hemizygous mice also included predominantly blastlike and myelo-monocytic cells (Figure 2.4, top row) with the characteristic cell-surface profile c-kit$^+$/ Lin$^-$ (Figure 2.5A). The peripheral blood smears, stained with Wright Giemsa, also showed severe anemia characterized by a significant decrease in the number of red blood cells (Figure 2.4, second row)

The bone marrow of the leukemic mice was also similarly affected between the heterozygous and hemizygous restored mice. Histology analysis shows an infiltration of immature cells into the bone marrow, causing a decrease in the number of fat cells and the number of red blood cells and capillaries running through the bone marrow (Figure 2.4, third row). The heterozygous restored leukemic mice had a decrease in the number of megakaryocytes seen in the bone marrow, and those that were present were immature (Kuo et al, 2006). The hemizygous restored leukemic mice had no observable megakaryocytes in the bone marrow, which again suggests that the fusion protein has an

Figure 2.4. Pathology of disease in *Cbfb*$^{+/MYH11}$ and *Cbfb*$^{-/MYH11}$ leukemic mice. Top row- Peripheral blood smears stained with Wright-Giemsa (1000X mag) **Second row-** Peripheral blood smears stained with Wright-Giemsa (400X). **Third row-** Bone marrow sections from tibia stained with H&E (400X) **Fourth row-** Disruption of spleenic architecture (400X) Spleen sections stained with H&E. **Bottom row-** The presence of infiltrating leukemia cells in the liver (400x) Liver sections stained with H&E. Cells analyzed from wild type control (left column), and leukemic mice expressing Cbfβ-SMMHC in the presence (middle column) or absence (right column) of Cbfβ.

Figure 2.4


Cbfb$^{+/+}$
Cbfb$^{+/MYH11}$
Cbfb$^{-/MYH11}$
Peripheral Blood Smear 1000X
Peripheral Blood Smear 400X
Bone Marrow 400X
Spleen 400X
Liver 400X

incomplete block of CBF function and that the combined lack of Cbfβ makes this blockage complete.

Leukemic mice with the presence of the fusion also presented with severe splenomegaly. Although the average spleen weight for the hemizygous restored mice (0.85 ± 0.52 g) was lower than for the heterozygous restored mice (2.01 ± 0.76 g), it is still significantly greater than the average spleen weight of a healthy mouse (0.06 ± 0.01 g). The lower spleen weight is most likely due to the shorter latency period, which does not allow as much time for the spleen to become infiltrated and enlarged. Histology analysis of the spleen confirms the disruption of normal splenic architecture. The displacement of the normal population of B-cells and T-cells by the infiltrating immature cells causes a lack of organized white pulp and a decrease in the amount of red pulp. (Figure 2.4, fourth row).

Histological analysis of the liver showed an infiltration of leukemic cells into the interstitial spaces and between the hepatocytes focused near the capillaries (Figure 2.4, bottom row). Compromise of the thymus and lymph nodes was not observed, which supports the conclusion that this is a myeloid and not a lymphoid disease. Taken together, the pathology of disease in hemizygous and heterozygous mice was similar, suggesting that the AML phenotype was determined by the presence of Cbfβ-SMMHC.

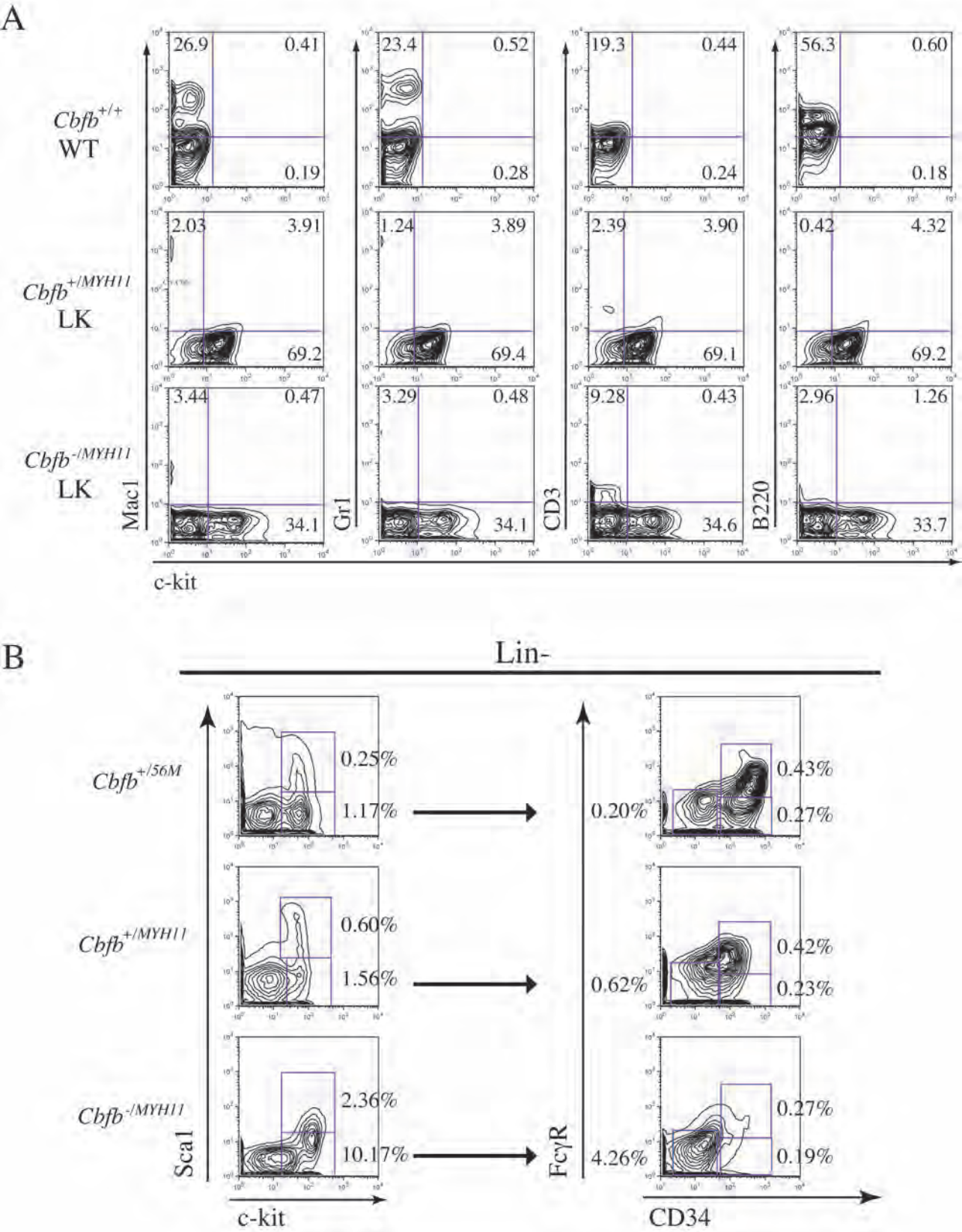
Peripheral blood from *Cbfb*$^{+/MYH11}$ and *Cbfb*$^{-/MYH11}$ leukemic mice shows a decrease in differentiated cells and an increase in progenitor cells. To further characterize the leukemic cells, flow cytometry was performed on the peripheral blood from the leukemic mice at the time of sacrifice (See Materials and Methods). Heterozygous and

hemizygous restored leukemic mice both had a decrease in the percentage of differentiated cells- $Gr1^+$ (granulocytes), $Mac1^+$ (macrophages), $CD3^+$ (T-cells), and $B220^+$ (B-cells) and an increase in the percentage of c-kit^+ cells in the peripheral blood (Figure 2.5A). This represents an overall increase in progenitor cells and a decrease in the percentage of differentiated myeloid and lymphoid cells, which suggests a block in differentiation.

Bone marrow from *Cbfb*$^{+/MYH11}$ and *Cbfb*$^{-/MYH11}$ pre-leukemic mice shows an increase in myeloid progenitor cells. To further examine the *in vivo* effect of the lack of Cbfβ in mice with the fusion protein, a pre-leukemic analysis was done on the bone marrow of these mice. The myeloid progenitor compartment was analyzed by flow cytometry using the progenitor markers Sca1 and c-kit (Akashi et al, 2000). In both the heterozygous and hemizygous pre-leukemic mice there was a small increase in the HSC population ($Lin^-/Sca1^+/c\text{-}kit^+$) and a dramatic (~20 fold) increase in the myeloid progenitor population ($Lin^-/Sca1^-/ckit^+$) (Figure 2.5B). This effect was similar to that seen by Kundu et al (2003) in *Cbfb*$^{GFP/GFP}$ embryos and by Miller et al (2002) in *Cbfb*$^{-/-}$*;Tek-Cbfb* rescued embryos (Kundu & Liu, 2003; Miller et al, 2002). The myeloid progenitor population was then sorted with the early progenitor markers FcγR and CD34 (Akashi et al, 2000). The granulocyte/macrophage progenitor (GMP: $Fc\gamma R^+/CD34^+$) and common myeloid progenitor (CMP: $Fc\gamma R^-/CD34^+$) populations changed very little compared to the wild-type control mice. However the megakaryocyte/erythroid progenitor (MEP: $Fc\gamma R^-/CD34^-$) population had a very significant increase in both of the pre-leukemic genotypes (Figure 2.5B). These progenitor cells from the *Cbfb*$^{+/MYH11}$ mice

Figure 2.5. Flow cytometry analysis of *Cbfb*$^{+/MYH11}$ and *Cbfb*$^{-/MYH11}$ leukemic and pre-leukemic mice. **A:** Flow cytometry analysis of peripheral blood from wild-type and leukemic *Cbfb*$^{+/MYH11}$ and *Cbfb*$^{-/MYH11}$ mice using lineage markers Mac1 (macrophages), Gr1 (granulocytes), CD3 (T-cells), and B220 (B-cells) and the progenitor marker c-kit. **B:** Flow cytometry analysis of bone marrow from wild-type and pre-leukemic *Cbfb*$^{+/MYH11}$ and *Cbfb*$^{-/MYH11}$ mice looking at Lin- cells using progenitor markers Sca1 and c-kit to examine hematopoietic stem cells and myeloid progenitor cells, and early progenitor markers FcγR and CD34 to examine MEPs, CMPs, and GMPs.

Figure 2.5


A
Cbfb+/+
WT
Cbfb+/MYH11
LK
Cbfb-/MYH11
LK
Mac1
Gr1
CD3
B220
c-kit
26.9
0.41
0.19
23.4
0.52
0.28
19.3
0.44
0.24
56.3
0.60
0.18
2.03
3.91
69.2
1.24
3.89
69.4
2.39
3.90
69.1
0.42
4.32
69.2
3.44
0.47
34.1
3.29
0.48
34.1
9.28
0.43
34.6
2.96
1.26
33.7
B
Lin-
Cbfb+/56M
Cbfb+/MYH11
Cbfb-/MYH11
Sca1
c-kit
FcγR
CD34
0.25%
1.17%
0.60%
1.56%
2.36%
10.17%
0.20%
0.43%
0.27%
0.62%
0.42%
0.23%
4.26%
0.27%
0.19%

are the abnormal myeloid progenitors (AMPs) seen in Kuo et al (2006) (Kuo et al, 2006), which suggests that the progenitor cells from the *Cbfb*$^{-/MYH11}$ mice are also AMPs.

The wild type *CBFB* allele is not a frequent target of mutations in inv(16) AML. The results obtained in the mouse prompted us to investigate whether *CBFB* was altered in human *CBFB/MYH11* AML samples. First, sequence analysis of the *CBFB* coding region in a panel of 29 inv(16) AML samples identified no mutations, in concordance with a previous report in 2 inv(16) AML samples (Leroy et al, 2002). In addition, analysis of *CBFB* transcript levels in a panel of 285 human AML samples indicated that inv(16) AML samples had a reduction of 40% of the *CBFB* transcript when compared to CD34^{+} BM cells (relative value: 0.4±0.08), as expected by the expression of one *CBFB* allele. The *CBFB* levels in t(8;21) and non-CBF cytogenetic groups were unchanged (t(8;21) relative value = 0.9±2.3; non-CBF relative value = 1.0±0.32). These results indicate that a wild type *CBFB* transcript may be expressed at reduced levels in inv(16) AML cells as a consequence of the inv(16), but additional genetic alterations are not common in the *CBFB* locus. The possible role of CBFβ as a tumor suppressor in human leukemia by post-transcriptional regulation mechanisms, however, cannot be ruled-out.

Discussion

Endogenous expression of Cbfβ-SMMHC from the *Cbfb* allele in conditional knock-in mice is a "class II" mutation, which creates an AMP cell that is able to progress to AML in a multistep process (Kuo et al, 2006). Although the fusion protein is thought to act as a dominant factor in differentiation and transformation (Castilla et al, 1999; Castilla et al, 1996; Kuo et al, 2006), the role of wild-type Cbfβ in Cbfβ-SMMHC mediated leukemia is not clear. Here we showed that the capacity of Cbfβ-SMMHC to induce AML in mice is modulated by wild-type Cbfβ *in vivo*.

Previous studies have shown that Cbfβ is critical for embryonic definitive hematopoiesis (Wang et al, 1996b), and for *in vitro* myeloid differentiation from *Cbfb*$^{-/-}$ embryonic stem cells (Miller et al, 2001). This study indicates that Cbfβ is necessary for *in vitro* myeloid-erythroid differentiation of BM-hematopoietic progenitors. In addition, since colonies were drastically reduced in the absence of Cbfβ, but not in the presence of Cbfβ-SMMHC, these results support the hypothesis that Cbfβ-SMMHC may have an incomplete effect in differentiation. As Cbfβ and Cbfβ-SMMHC compete for binding with Runx proteins in BM cells, basal levels of the Cbfβ:Runx1 complex in hematopoietic progenitors expressing Cbfβ-SMMHC may be critical for proliferation of myeloid progenitors and result in a delay in transformation.

In vitro data does not show *Cbfb*$^{-/MYH11}$ cells with an increased ability to proliferate. Instead, the increase in progenitor cells provides a larger more susceptible pool of cells that are prone to acquiring proliferative mutations. The full block in differentiation may cause acceleration of this natural acquisition of genetic aberrations.

Endogenous expression of Cbfβ-SMMHC and Cbfβ in BM induces AML with a median latency of 5 months (Kuo et al, 2006). Upon the Cre-lox-mediated switch from Cbfβ to Cbfβ-SMMHC expression in progenitor cells lacking a wild type *Cbfb* allele, AML latency was shortened to 6 weeks. This finding strongly suggests that Cbfβ-SMMHC function is enhanced by wild-type Cbfβ loss.

The *Cbfb*$^{-/MYH11}$ mice studied here present with all the characteristics of leukemia. White blood cell counts are significantly increased and peripheral blood smears show severe anemia. Flow cytometry analysis of the peripheral blood shows a block in the differentiation of hematopoietic cells including granulocytes, macrophages, T-cells, and B-cells. There is also an increase in the number of myeloid progenitor cells, which look immuno-histochemically like MEPs but morphologically may represent CMPs, MEPs, or GMPs. Histological analysis of hematopoietic tissues shows infiltration of immature leukemic cells in the bone marrow, spleen, and liver, but not the thymus or lymph nodes. Lastly, bone marrow and spleen cells from *Cbfb*$^{-/MYH11}$ leukemic mice are transplantable into wild-type recipient mice with latency similar to that of *Cbfb*$^{+/MYH11}$ leukemic mice.

In *Cbfb*$^{-/MYH11}$ leukemic mice there is a smaller increase in the number of white blood cells in the peripheral blood, compared to the leukemic *Cbfb*$^{+/MYYH11}$ mice. In *Cbfb*$^{+/MYYH11}$ mice, it is probable that the wild-type copy of *Cbfb* provides a partial rescue of the differentiation block. In *Cbfb*$^{-/MYYH11}$ mice, a wild-type copy of *Cbfb* is not present to rescue the block in differentiation in the hematopoietic cells; therefore there is more of a complete block in differentiation. As in the less severe splenomegaly, time may also be

a factor in accumulation of white blood cells. Because of the shorter latency, there is not as much time to amass more white blood cells.

Normally, the number of colonies at day 7 reflects the number of progenitor cells plated. Because Cbfβ-SMMHC causes a block in differentiation, an increase in the number of progenitor cells and therefore the number of colonies from Cbfβ-SMMHC expressing cells would be expected. This is the case for the *Cbfb*$^{+/MYH11}$ pre-leukemic bone marrow, however there is a decrease in the number of colonies from the *Cbfb*$^{-/MYH11}$ pre-leukemic bone marrow. In the *Cbfb*$^{-/MYH11}$ expressing cells, there are two possible explanations for decrease in colony number. First, is possible that there is actually a decrease in the number of progenitor cells with this genotype. This is unlikely, since the presence of the fusion protein alone causes an increase in the number of progenitors due to a block in differentiation and the lack of *Cbfb* does not reverse that effect. The other possibility is that there are progenitor cells present, but that they are unable to proliferate adequately to create a large enough colony to count. The relatively small and less dense colonies seen in the *Cbfb*$^{-/MYH11}$ cells support this latter hypothesis. *In vitro* over-expression experiments also show that CBFβ-SMMHC slows cell proliferation at the G1 to S cell cycle transition (Cao et al, 1998; Cao et al, 1997).

Cytological analysis of the pre-leukemic bone marrow showed the expected increase in blast colonies in those colonies containing Cbfβ-SMMHC, although in the absence of Cbfβ there is a slightly greater increase in the percentage of blast colonies. This suggests a greater block in differentiation in the *Cbfb*$^{-/MYH11}$ mice. Those colonies expressing Cbfβ-SMMHC and lacking wild-type Cbfβ showed significant decreases in

the percentage of CFU-G and CFU-GM colonies compared to controls. Those colonies with both the fusion protein and wild-type Cbfβ showed significant increases in CFU-G and decreases in CFU-M. This suggests that the differentiation block may be slightly different between these two genotypes, and that the presence of the fusion protein in pre-leukemic bone marrow cells causes a block in differentiation at the CMP level, an increase in progenitor cells, and no significant change in proliferation.

Surprisingly, a similar AML latency was observed between induced and uninduced groups. Probably, a small progenitor population underwent Cre/lox deletion due to ‘‘leaky’’ Cre expression from the *Mx1Cre* transgene (Chan et al, 2004), and thus expressed the *Cbfb/MYH11* fusion gene. Importantly, all AML samples presented the Cre mediated deletion, suggesting that transformation is due to the Cbfβ to Cbfβ-SMMHC switch. Furthermore, the finding that *CBFB* is not frequently lost in human AML argues against its role as an inv(16) cooperating tumor suppressor in AML. Rather, our results suggest that an increase in the Cbfβ-SMMHC to Cbfβ ratio reduced proliferation of myeloid progenitors while increasing their susceptibility to neoplastic transformation, although the underlying mechanism is unclear. However, this study cannot rule out the possibility that *Cbfb* loss in bone marrow could induce AML. The generation of conditional *Cbfb* knockout alleles will provide a critical tool to directly address this possibility using a genetic approach.

Runx2 has previously been shown to cooperate with *Cbfb/MYH11* to cause leukemia (Castilla et al, 2004). This result presents a novel idea, because both mutations, a block in differentiation and an increase in proliferation, occur in the same pathway. Together, these results highlight the importance of the CBF transcription factor in

regulation of hematopoiesis and contribution to leukemogenesis. Finally, these findings have important implications on the design of targeted therapies. One possibility is the identification of drugs that inhibit the fusion protein. Whereas candidate molecules should act to disrupt Cbfβ-SMMHC:Runx1 binding, it will be critical that CBFβ:Runx1 binding remains unaltered.

CHAPTER III

ABROGATION OF THE POSTMITOTIC CHECKPOINT CONTRIBUTES TO POLYPLOIDIZATION IN E7 EXPRESSING, RB-DOWNREGULATED CELLS

Preface

All of the experiments in this chapter were done by the author with the exception of the time-lapse videomicroscopy, which was done by Joshua Nordberg, in the laboratory of Greenfield Sluder, Department of Cell Biology, University of Massachusetts Medical School and analyzed by the author. The work in this section has been submitted for publication:

Heilman SA, Nordberg JJ, Liu YW, Sluder G, Chen JJ. HPV-E7 induces polyploidy through abrogation of a novel post-mitotic checkpoint.

Abstract

Polyploidy predisposes human cells to aneuploidy, an early event in multistep carcinogenesis. Cells with defective retinoblastoma (Rb) protein, which is functionally inactivated in the majority of human cancers, have a high incidence of polyploidy. However, the mechanism of polyploidization in Rb-downregulated cells is not known, and current hypotheses are conflicting. Previous proposed mechanisms include spindle assembly checkpoint abrogation, DNA re-replication without an intervening mitosis, cleavage failure, and/or a relaxed postmitotic checkpoint. This study explores these possibilities to better characterize the mechanism by which Rb-downregulation leads to polyploidization. We find that Rb-defective mouse and human cells undergo normal mitoses with an intact spindle assembly checkpoint and complete cytokinesis. Our results also exclude DNA re-replication as a major mechanism of polyploidization in Rb-downregulated cells upon microtubule disruption. While normal cells arrest in G1 with 4C DNA content after adaptation to the spindle assembly checkpoint, Rb-downregulated cells replicate their DNA after mitosis and propagate as polyploid cells. Thus, our data indicate that Rb-downregulation leads to abrogation of the postmitotic checkpoint, which allows polyploidization.

Introduction

An important hallmark of human cancers is aneuploidy, the state in which a cell has extra or missing chromosomes (Nasmyth, 2002; Pellman, 2007). Polyploidy, the state in which cells have more than two sets of chromosomes, is thought to be an early event in multi-step carcinogenesis that can lead to aneuploidy (Andreassen et al, 2001; Meraldi et al, 2002), as exemplified in Barrett's esophagus (Galipeau et al, 1996). Polyploidy has recently been shown to not only occur as an early event in cervical carcinogenesis, but to predispose the cells to aneuploidy (Olaharski et al, 2006). Other recent studies have shown that tetraploid, but not diploid, mouse or human cells induce tumor formation in mice (Duelli et al, 2007; Fujiwara et al, 2005). These studies highlight the potential importance of polyploidy in carcinogenesis.

The retinoblastoma (Rb) tumor suppressor protein is functionally inactivated in the majority of human cancers (Hanahan & Weinberg, 2000). Rb blocks proliferation of cells by sequestering members of the E2F family of transcription factors, which are essential for G1 to S-phase progression (Weinberg, 1995). Downregulation of Rb has been shown to spontaneously induce polyploidy in cycling cells (Hernando et al, 2004; Iovino et al, 2006) or in cells under mitotic stress caused by nocodazole or colcemid (Di Leonardo et al, 1997; Khan & Wahl, 1998; Lentini et al, 2002), suggesting that pRb may be a major regulator of the mechanism that controls cellular ploidy (Lentini et al, 2002).

The cellular mechanisms responsible for this polyploidization are as yet undetermined, but several models have been proposed. When cells with an intact spindle assembly checkpoint become arrested during mitosis for a prolonged period of time, they eventually adapt to the checkpoint, exit mitosis without cleavage, and progress into a G1-

like state with 4C DNA content (Lanni & Jacks, 1998; Margolis et al, 2003). The cells are prevented from continuing through the cell cycle and replicating their DNA by a proposed p53- and pRb-dependent postmitotic checkpoint (Khan & Wahl, 1998; Lanni & Jacks, 1998). The first model proposes that abrogation of this checkpoint allows the cells to replicate their 4C DNA leading to proliferation of the polyploid cells. This has been shown in cells that express the HPV-16 E6 oncogene that degrades p53 (Liu et al).

A second proposed model for polyploidization in Rb-downregulated cells is re-replication, a process of multiple rounds of DNA replication without an intervening mitosis. It was previously proposed that Rb is involved in preventing DNA re-replication and that downregulation of Rb may allow cells to become polyploid (Lentini et al, 2002). Another proposal is abrogation of the spindle assembly checkpoint followed by cleavage failure, which may lead to polyploidization (Taylor & McKeon, 1997; Vogel et al, 2004) in Rb-downregulated cells (Thomas & Laimins, 1998). Hernando et al (2004) suggested that Rb-downregulation affects the spindle checkpoint through upregulation of Mad2 due to deregulation of E2F activity (Hernando et al, 2004). Finally, cleavage failure, which yields binucleate cells with 4C DNA content, is also a potential mechanism to polyploidization (Shi & King, 2005). In this study, I investigate these possibilities to determine the mechanism that leads to polyploidization in Rb-downregulated cells.

Materials and Methods

Cell Culture. Mouse embryonic fibroblasts (MEFs) (provided by Stephen Jones, Department of Cell Biology, University of Massachusetts Medical School) were maintained in Dulbecco's modified Eagle medium (DMEM) plus 15% fetal bovine serum (FBS). Primary human keratinocytes (PHKs) were derived from neonatal human foreskin epithelium obtained from the University of Massachusetts Hospital as described (Liu et al). These cells were maintained on mitomycin C-treated J2-3T3 feeder cells in F-medium composed of 3 parts Ham's F12 medium and 1 part DMEM plus 5% FBS with all supplements as previously described (Flores et al, 2000). The hTERT-expressing human retinal pigment epithelial cells (RPE1) (Uetake & Sluder, 2004) were maintained in 1:1 DMEM and Ham's F12 medium plus 10% FBS.

Retroviral Infections. PHKs and RPE1 cells expressing vector or E7 were established by retrovirus-mediated infection using the pBabe-puro-based retroviral construct. After puromycin selection (0.75 μg/mL for PHKs and 10 μg/mL for RPE1 cells), populations of infected cells were pooled. PHKs were maintained in 0.2 μg/mL puromycin and used within 5 passages. RPE1 cells were maintained in 2 μg/mL puromycin and used within 8 passages. E7 expression was confirmed by RT-PCR using previously described oligos (Liu et al) and by Western blot.

Immunoblotting. Protein extraction was performed in RIPA lysis buffer (150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, 5mM EDTA, protease inhibitors (Complete Mini EDTA-free, Roche)). Protein concentrations were determined

by bicinchoninic acid (BCA) analysis (Pierce). Equal amounts of protein from each cell lysate were separated by SDS-PAGE and transferred onto a PVDF membrane. The membranes were blotted with antibodies against E7 (Santa Cruz), pRb (BD Biosciences), cyclin B1 (BD Biosciences), cyclin D1 (Santa Cruz), cyclin E (BD Biosciences), and β-tubulin (Sigma). Immunoreactive proteins were visualized with SuperSignal® West Femto Maximum Sensitivity Substrate (Pierce). The membranes were scanned with an LAS-1000 Image Reader (Fuji Photo Film Inc.).

Flow cytometry. Asynchronous cultures of cells were treated with DMSO (Sigma), nocodazole (Sigma, 50 ng/mL), or Monastrol (Sigma, 100 μM). For the polyploidy experiments, the cells were harvested, fixed in 70% ethanol, and resuspended in a PBS-propidium iodide (PI, Sigma, 50 μg/mL)-RNase A (Sigma, 70 μg/mL) solution. For the mitotic index and mitotic shake-off experiments, the cells were harvested at 12, 16, 20, 24, 28, 40, 42, 48 or 72 hours, fixed in 70% ethanol, permeablized in 0.25% Triton X-100, stained with 0.5 μg of the rat anti-phospho-histone H3 IgG_{2a} (Sigma) and then with fluorescein isothiocyanate (FITC)-conjugated anti-rat IgG_{2a} (BD Biosciences), and counter-stained with PBS-PI-RNase A. For the BrdU labeling experiments, the plated cells were incubated with BrdU (final 10 μM) for 2 hours before being harvested and fixed in 70% ethanol. After fixation, the cells were permeablized with 2 N HCl/ 0.5% Triton X-100, neutralized with 0.1 M sodium tetraborate, stained with anti-BrdU-FITC (BD Biosciences), stained with anti-mouse IgG $(Fab)_2$-FITC (Sigma), and counter-stained with PBS-PI-RNase A. Cell cycle analysis was performed using FlowJo software (Becton Dickinson).

Time-Lapse Videomicroscopy. Live-cell imaging was performed as previously described (Sluder et al, 2004). Briefly, RPE1 cells or MEFs were grown on glass 22 x 22 mm coverslips in low HEPES (12.5 mM) DMEM for time-lapse videomicroscopy. Phase contrast images were acquired on a Leica DMIRE2 inverted microscope with a 10x objective lens, and images were acquired using Openlab 3.5.2 (Improvision, Inc., Lexington, MA) and recorded using a Q-Imaging Retiga Exi camera. Images were taken every 3 minutes with a 3 second exposure and later compressed to view as a movie using Quicktime 6.5 (Apple Computer, Inc., Cupertino, CA).

Statistical Analysis. Data were expressed as mean ± standard deviation. Differences between means were assessed by Student's t-test. $p \leq 0.05$ was considered significant.

Results

Rb downregulation results in polyploidy. It has been shown that downregulation of Rb in mouse and human fibroblasts leads to spontaneous polyploidization (Hernando et al, 2004; Iovino et al, 2006). We first tested these results in our own cells by comparing $Rb^{-/-}$ MEFs to wild-type MEFs. As shown in Figure 3.1, $Rb^{-/-}$ MEFs have 5-fold more cells with DNA content greater than 4C compared to wild-type controls (14.5% vs. 3.0%). Previous studies showed that treatment with nocodazole, which disrupts microtubule stability, enhances polyploidization in $Rb^{-/-}$ MEFs (Di Leonardo et al, 1997; Khan & Wahl, 1998; Lentini et al, 2002). Our study also showed that arresting $Rb^{-/-}$ MEFs in mitosis with nocodazole, where they eventually adapt to the spindle assembly checkpoint, results in double the amount of polyploidy compared to untreated cells (28.3% vs. 14.5%, Figure 3.1). This confirms that lack of Rb in MEFs can lead to polyploidy in our cells, both spontaneously and in response to microtubule depolymerizing drugs.

Next we examined the effect of Rb-downregulation in human epithelial cells, the tissue type from where the majority of human solid tumors arise (Christofori & Semb, 1999). Since no matched human cell lines defective for Rb are available, we used the human papillomavirus type 16 (HPV-16) oncogene E7 to downregulate pRb. HPV-16 E7 is known to bind pRb and promote its degradation (Demers et al, 1994; Dyson et al, 1989). Stable cell lines were created by infection of both primary human keratinocytes (PHKs) and hTERT-immortalized human retinal pigment epithelial (RPE1) cells with retroviruses containing E7 or empty vector. E7 expression in the stable cell lines was confirmed by RT-PCR (Figure 3.2A) and by Western blot (Figure 3.2B). As

Figure 3.1. Rb downregulation results in polyploidy formation. Asynchronous cultures of wild-type and Rb-/- MEFs were treated with DMSO or nocodazole (50 ng/mL) for 48 hours.

Figure 3.1

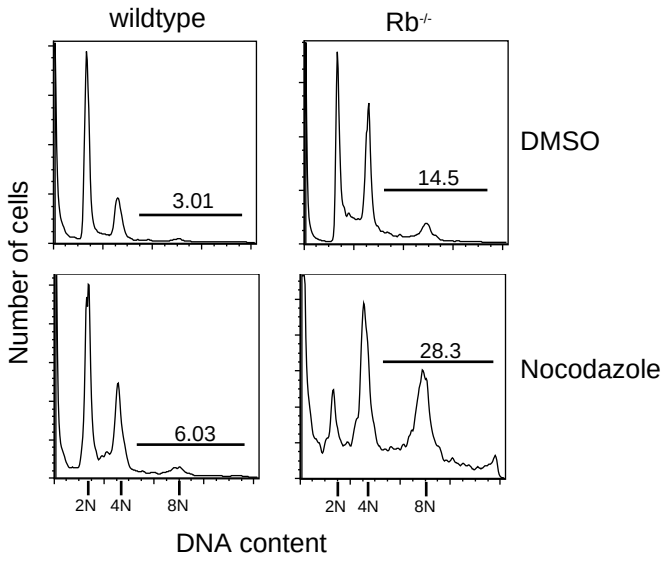
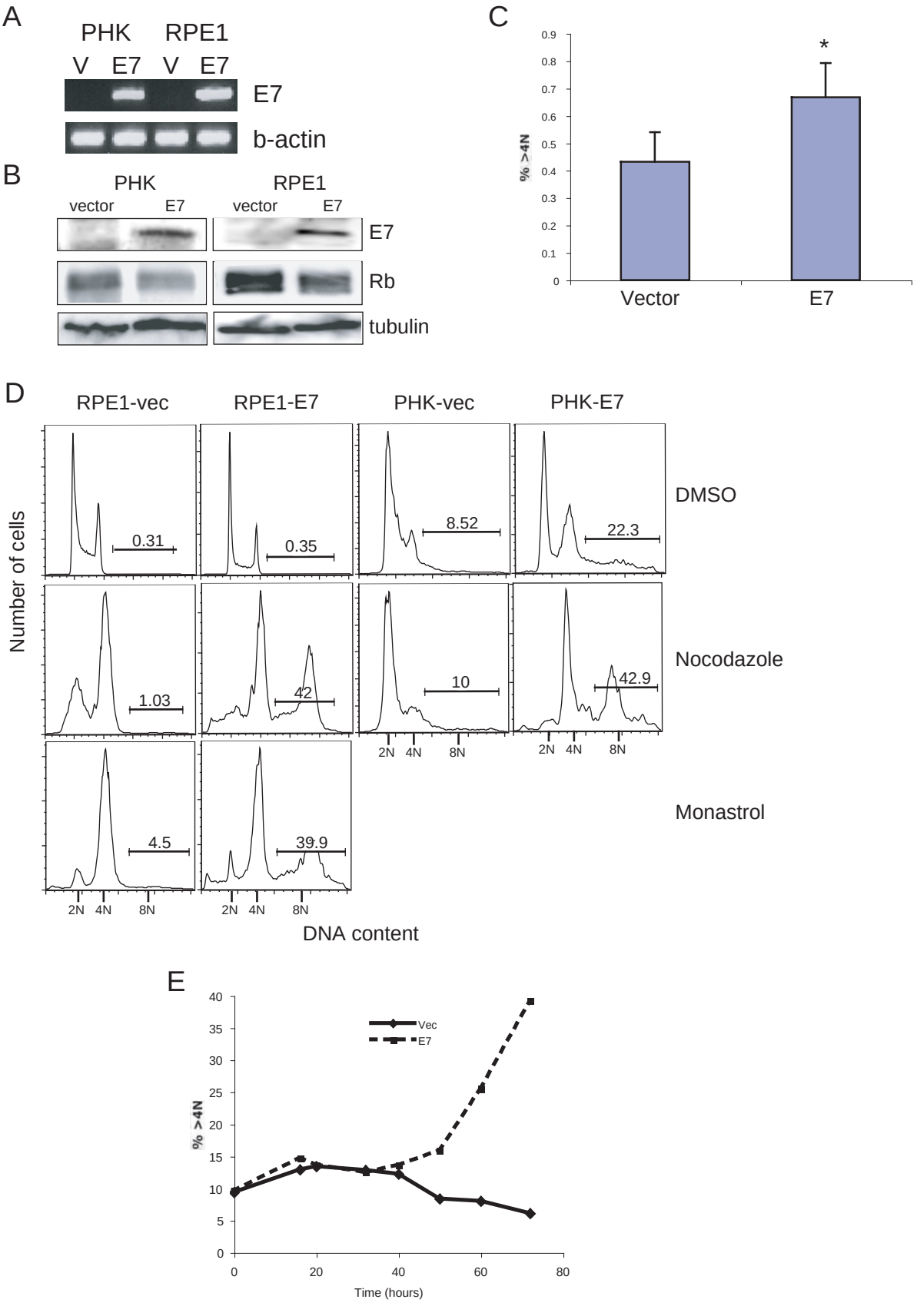

wildtype
Rb-/-
DMSO
Nocodazole
3.01
14.5
6.03
28.3
Number of cells
2N
4N
8N
2N
4N
8N
DNA content

Figure 3.2. E7 expression results in polyploidy formation. A. Total RNA isolated from PHKs and RPE1 cells expressing vector or E7 were subjected to RT-PCR using specific primers. The products were resolved on a 1% agarose gel. β-actin was used as a control. **B.** Total protein extracts from PHKs and RPE1 cells expressing vector or E7 were resolved by SDS-PAGE and blotted with antibodies against E7, pRb, and tubulin. **C.** Untreated RPE1 cells expressing E7 or vector were harvested, fixed, and stained with PI. The percentages of polyploidy (>4C DNA content) was measured and averaged. (* $p<0.001$) **D**. Asynchronous cultures of RPE1 cells and PHKs expressing vector or E7 were treated with DMSO, nocodazole (50 ng/mL), or Monastrol (100 μM). Seventy-two or forty-eight hours later, the RPE1 cells and PHKs, respectively, were collected, fixed, stained with PI, and analyzed by flow cytometry. Data from a representative experiment (of 3) are shown. The percentages of cells with more than 4C DNA content are indicated. **E.** Cells under nocodazole treatment (50 ng/mL) were harvested at various time points, fixed, stained with PI, and analyzed by flow cytometry. The percentage of cells with DNA content greater than 4C was plotted as a function of time.

Figure 3.2


A
PHK
RPE1
V E7 V E7
E7
b-actin
B
PHK
RPE1
vector E7 vector E7
E7
Rb
tubulin
C
% >4N
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
*
Vector
E7
D
RPE1-vec
RPE1-E7
PHK-vec
PHK-E7
Number of cells
DMSO
Nocodazole
Monastrol
0.31
0.35
8.52
22.3
1.03
42
10
42.9
4.5
39.9
2N 4N 8N
DNA content
E
% >4N
Vec
E7
40
35
30
25
20
15
10
5
0
0 20 40 60 80
Time (hours)

expected, the steady-state levels of pRb is reduced in E7 expressing PHKs and RPE1 cells (Figure 3.2B). To examine the incidence of polyploidization over an extended period of time, wild-type and E7-expressing RPE1 cells were serially passaged for longer than one month and stained with PI as described (Materials and Methods) and analyzed by FACS for DNA content. The RPE1 cells were used for this experiment, because PHKs cannot be cultured for more than a few passages. Figure 3.2C shows that there was a small but statistically significant increase ($p < 0.001$) in the percentage of polyploid cells in the Rb-downregulated RPE1-E7 cells ($0.67\% \pm 0.13\%$) compared to the RPE1-vector control cells ($0.43\% \pm 0.11\%$).

Although $Rb^{-/-}$ MEFs have a higher incidence of spontaneous polyploidization than E7-expressing RPE1 cells, these differences can be attributed to several factors. In MEFs, which as primary cells tend to acquire more mutations in culture, the Rb downregulation is not as complete in E7 transfected RPE1 cells as it is in $Rb^{-/-}$ MEFs. Also, E7 has been shown to have Rb-independent biological activities (Munger et al, 2001), such as interactions with cell cycle regulators such as p21, p27, and cdc25A, which may cause other effects on the cell (Funk et al, 1997; Katich et al, 2001; Zerfass-Thome et al, 1996).

It has previously been thought that prolonged activation of the spindle assembly checkpoint is necessary for the mechanism that leads to polyploidy. The low incidence of spontaneous polyploidy may have been because untreated cells trigger the spindle assembly checkpoint only during rare spontaneous events. In order to increase the frequency with which the cells have prolonged activation of the spindle assembly checkpoint, the microtubule depolymerizing drug nocodazole was used. After treating

with nocodazole, E7-expressing PHKs and RPE1 cells showed a significant increase in polyploidy compared to vector controls (42.9% vs 10.0% in PHKs and 42.0% vs 1.0% in RPE1 cells, Figure 3.2D). Time course analysis of polyploidization indicates that the increase in polyploidy in RPE1-E7 cells begins approximately 40 hours after nocodazole treatment (Figure 3.2E). To investigate to what extent this result is specific to nocodazole, the drug Monastrol, a small molecule inhibitor of the mitotic kinesin motor protein Eg5, which triggers the spindle assembly checkpoint through formation of monopolar spindles, was used. The RPE1-E7 cells also showed an increase in polyploidy (39.9% vs 4.5%) in response to Monastrol (Figure 3.2D).

Rb downregulation does not effect the spindle assembly checkpoint or mitosis. A number of studies have proposed that downregulation of Rb contributes to loss of spindle assembly checkpoint control and that this may be a mechanism for polyploidization (Hernando et al, 2004; Patel et al, 2004; Thomas & Laimins, 1998). On the other hand, Hernando et al (2004) suggest that Rb-downregulation causes hyperactivation of the spindle checkpoint through upregulation of MAD2 (Hernando et al, 2004). However, these contradictory studies did not examine the effect of Rb-downregulation on the spindle assembly checkpoint directly, but rather used DNA content alone to examine the cell cycle. Here the effect of Rb on the checkpoint by using an antibody against the mitotic marker phospho-histone H3 (P-his H3) that stains mitotic cells from prophase to late telophase (Juan et al, 1998) was examined. Concurrent staining with both the P-his H3 antibody and PI allows differentiation between mitotic cells and interphase cells with a 4C DNA content. A mitotic index curve was constructed by treating wild-type and $Rb^{-/-}$

MEFs with nocodazole and examining the percentage of mitotic cells with a 4C DNA content. By staining the cells at various time points, the percentage of cells in mitosis over time was determined. This allowed for differentiation between spindle assembly checkpoint activation and abrogation. When the cells halt in mitosis, the percentage of cells positive for P-His H3 increases. As the cells adapt to the spindle assembly checkpoint, the percentage of cells positive for P-His H3 decreases. If the spindle assembly checkpoint were abrogated by downregulation of Rb, the percentage of cells in mitosis are expected to be significantly lower than in the control cells.

Figure 3.3A shows that the mitotic index curves for wild-type MEFs and $Rb^{-/-}$ MEFs are not significantly different. The percentage of cells halted in mitosis is relatively low in MEFs most likely because the cells do not grow very well. Therefore, in order to obtain larger numbers of cells in mitosis and to examine the spindle assembly checkpoint in human epithelial cells, mitotic index curves were also constructed for Rb-downregulated RPE1 cells and PHKs. Both of these cell types also showed no significant difference from controls (Figure 3.3B and C). Therefore the spindle assembly checkpoint is functional in Rb-downregulated cells, which suggests that an alternate mechanism leads to polyploidization in Rb-downregulated cells.

Time-lapse videomicroscopy was also used to compare the length of normal mitosis and cytokinesis for wild-type MEFs versus $Rb^{-/-}$ MEFs and RPE1-vector versus RPE1-E7 cells. The duration of mitosis was measured as the time between nuclear envelope breakdown (NEB) and nuclear envelope reformation (NER). Table 3.1 shows that the time spent in mitosis was not significantly different between wild-type MEFs and $Rb^{-/-}$ MEFs (41 min ± 11 min vs. 44 min ± 15 min) or RPE1-vector and RPE1-E7 cells

Figure 3.3. Rb-downregulation does not affect the spindle assembly checkpoint. Mitotic index of **(A)** wild-type and Rb-/- MEFs and **(B)** RPE1 cells and (**C)** PHKs expressing vector or E7. Cells under nocodazole treatment (50 ng/mL) were harvested at various time points, fixed, stained with rat anti-phospho-histone H3 IgG2a and FITC-conjugated anti-Rat IgG2a, stained with PI, and analyzed by flow cytometry. The percentage of cells positive for p-His H3 with a 4C DNA content was plotted as a function of time. Data represent the means of 2-3 determinants from a representative experiment of 3.

Figure 3.3

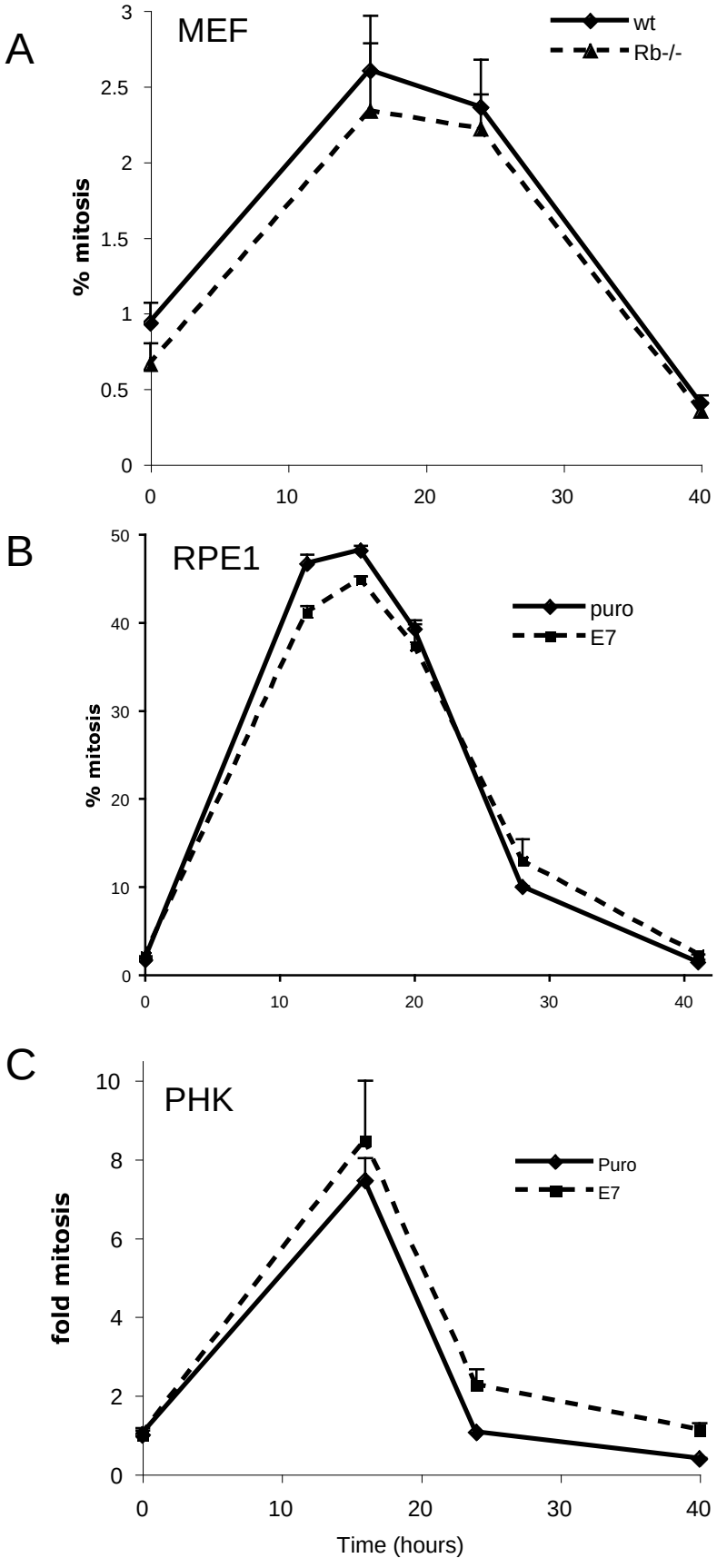

A
MEF
wt
Rb-/-
% mitosis
3
2.5
2
1.5
1
0.5
0
0
10
20
30
40
B
RPE1
puro
E7
% mitosis
50
40
30
20
10
0
0
10
20
30
40
C
PHK
Puro
E7
fold mitosis
10
8
6
4
2
0
0
10
20
30
40
Time (hours)

Table 3.1. Rb-downregulation does not affect mitosis as determined by time-lapse videomicroscopy.

Table 3.1. Rb-downregulation does not affect mitosis as determined by time-lapse videomicroscopy

	Number of cells	Mitosis (min)	Cytokinesis (min)	Mitosis + cytokinesis (min)
Wildtype MEFs	n=40	41±11	33±16	74±17
$Rb^{-/-}$ MEFs	n=40	44±15	45±27	89±38
p-value		0.40	0.01	0.03
RPE1-vector	n=56	34±7	96±28	128±30
RPE1-E7	n=17	37±6	102±39	138±40
p-value		0.19	0.66	0.54

(34 min ± 7 min vs. 37 min ± 6 min). These data are consistent with the mitotic index curves in Figure 3.3, supporting the hypothesis that lack of Rb does not have a significant effect on the duration of mitosis, an observation different from the previous study by Hernando et al (2004) (Hernando et al, 2004).

In agreement with what was described by Hernando et al (2004), the current time-lapse data show that $Rb^{-/-}$ MEFs did spend significantly more time completing cytokinesis than wild-type MEFs (Hernando et al, 2004). However, the cells did eventually cleave into two daughter cells. Therefore cleavage failure in response to spindle disruption does not appear to be a mechanism for polyploidization.

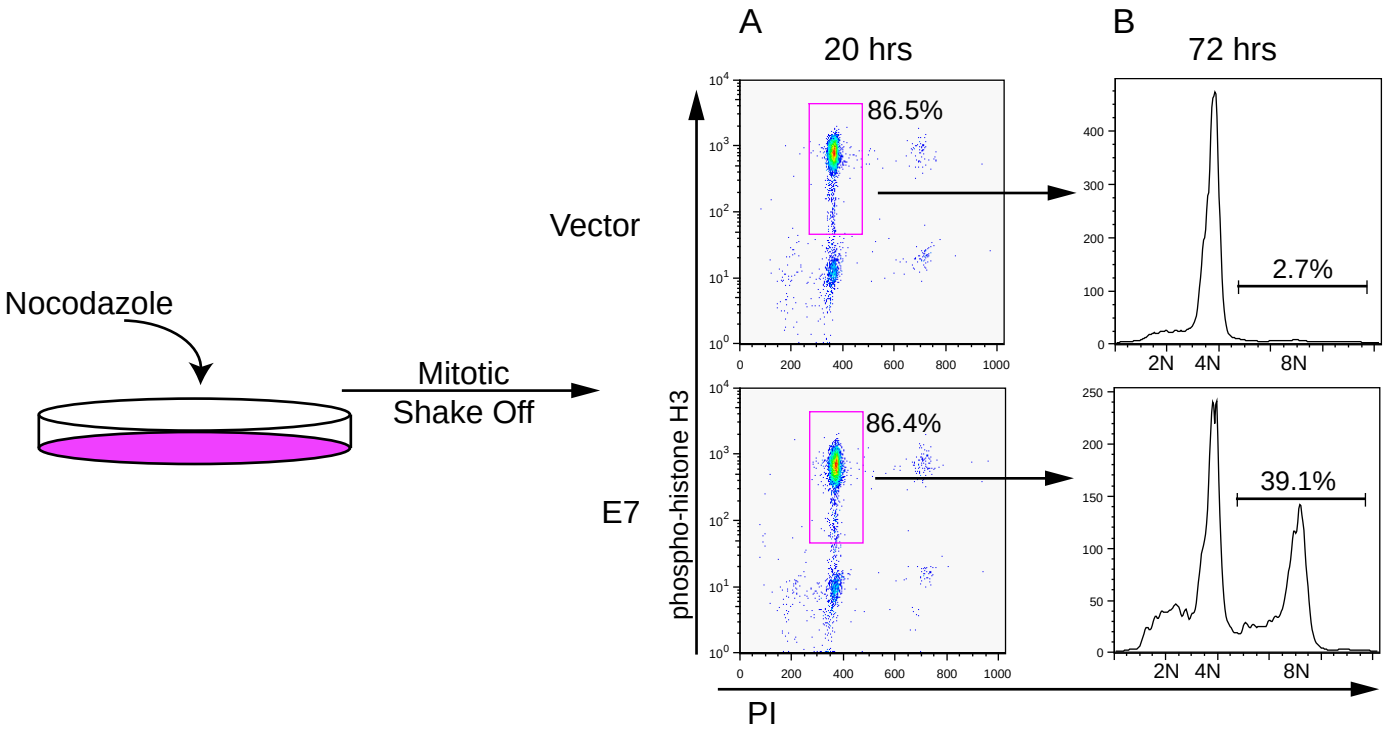
Re-replication is not a significant source of polyploidy in Rb-downregulated cells. It has been proposed that Rb plays a role in preventing DNA re-replication (Lentini et al, 2002). This possibility is directly tested here. In the $Rb^{-/-}$ MEFs and both PHKs and RPE1 cells expressing E7, cells exit mitosis approximately 16-20 hours after nocodazole treatment (Figure 3.3), but a significant incidence of polyploidy is not seen until approximately 40 hours after nocodazole treatment (Figure 3.2E). These cells have a 4C DNA content at mitosis yet do not show an 8C DNA content until 24 hours later. The kinetics of polyploidization in these Rb-downregulated cells suggest that re-replication of DNA does not contribute to polyploidization. Also, because re-replication is an inefficient process, the result is usually aneuploidy where the cell cycle profile would not have a discreet 8N peak, but rather a percentage of cells evenly spread out between 4N and 8N. The flow cytometric analysis has a discreet 8N peak, which suggests that the cells efficiently replicated its DNA, not by the process of re-replication.

In addition to this observation, we performed a mitotic shake-off experiment to examine the potential contribution of re-replication to polyploidization in Rb-downregulated cells. Cells that have entered mitosis "round-up" and become tenuously attached to the plate. Although some cells in prophase may decondense their chromosomes and return to interphase, the cells that are able to be shaken off the dish are considered to be at "a point of no return" and are committed to entering mitosis (Rieder & Cole, 1998). The mitotic shake-off experiment shown in Figure 3.4 was performed by first adding nocodazole to RPE1-vector or -E7-expressing cells. After 20 hours of nocodazole treatment, mitotic cells were shaken off and replated in nocodazole-containing media (Figure 3.4A). A majority of these shaken-off RPE1 cells (86%) were confirmed by flow cytometry to be in mitosis. With expression of E7, 39% of RPE1 cells became polyploid compared to only 2.7% of vector controls (Figure 3.4B). This demonstrates that E7 expressing polyploid cells can arise from a mitotic cell.

In order to further clarify the role of re-replication in E7-expressing cells, an all day mitotic shake-off experiment was also done. Nocodazole was added to RPE1 vector and E7 expressing cells, and starting 14 hours after treatment, mitotic cells were shaken off and collected every hour until 22 hours post-nocodazole treatment. All of the mitotic cells were pooled together and counted, and the remaining non-mitotic cells were trypsinized and counted. Approximately 76% of vector and 82% of E7-expressing cells entered mitosis during this 8 hour period. Under these conditions, 35-40% of RPE1-E7 cells would become polyploid. This suggests that many of these cells did enter mitosis before becoming polyploid, however it does not completely rule out re-replication as a mechanism to polyploidy. This experiment was only done over 8 hours, and more cells

Figure 3.4. Rb-downregulated mitotic cells are capable of polyploidy formation. Asynchronous RPE1 cells expressing vector or E7 were treated with nocodazole (50 ng/mL). **A.** After 20 hours of nocodazole treatment, cells were measured for p-His H3 expression and DNA content. Cells positive for p-His H3 with a 4C DNA content are indicated. Mitotic cells were shaken-off and replated in nocodazole-containing medium for an additional 52 hours. **B.** Cells were analyzed for PI content. Data from a representative experiment (of 3) are shown.

Figure 3.4


A
20 hrs
B
72 hrs
Vector
E7
Nocodazole
Mitotic
Shake Off
86.5%
86.4%
2.7%
39.1%
2N
4N
8N
phospho-histone H3
PI

may have continued to enter mitosis had the experiment been continued over a longer period of time (i.e. 16-24 hours). Also, an additional experiment that may help to further dismiss re-replication as a mechanism to polyploidy includes another type of mitotic shake-off experiment. After serially removing shake-off cells over an 8-12 hour period, the mitotic and non-mitotic cells would be stained with PI and analyzed by flow cytometry. If re-replication is not a major mechanism to polyploidization, we would expect to see very few cells with 8N DNA content from the E7 expressing non-mitotic cells.

Downregulation of Rb abrogates the postmitotic checkpoint. Next we tested whether abrogation of the postmitotic checkpoint after adaptation to the spindle assembly checkpoint contributes to polyploidization in Rb-downregulated cells. A previous study suggested that downregulation of Rb may facilitate DNA replication of cells with 4C DNA content in a G1-like state (Khan & Wahl, 1998). However, in this study, the cell cycle state and pRb level of the control cells after adaptation to mitotic disruption was not well characterized. Also, direct evidence of DNA replication in Rb-downregulated cells was not provided in this particular study. As a result, direct evidence of postmitotic checkpoint abrogation in Rb-downregulated cells leading to polyploidization has not been demonstrated. In order to study this proposed mechanism in more detail we created a cell population, confirmed it to be in a G1-like state, and examined the postmitotic checkpoint with a BrdU incorporation assay.

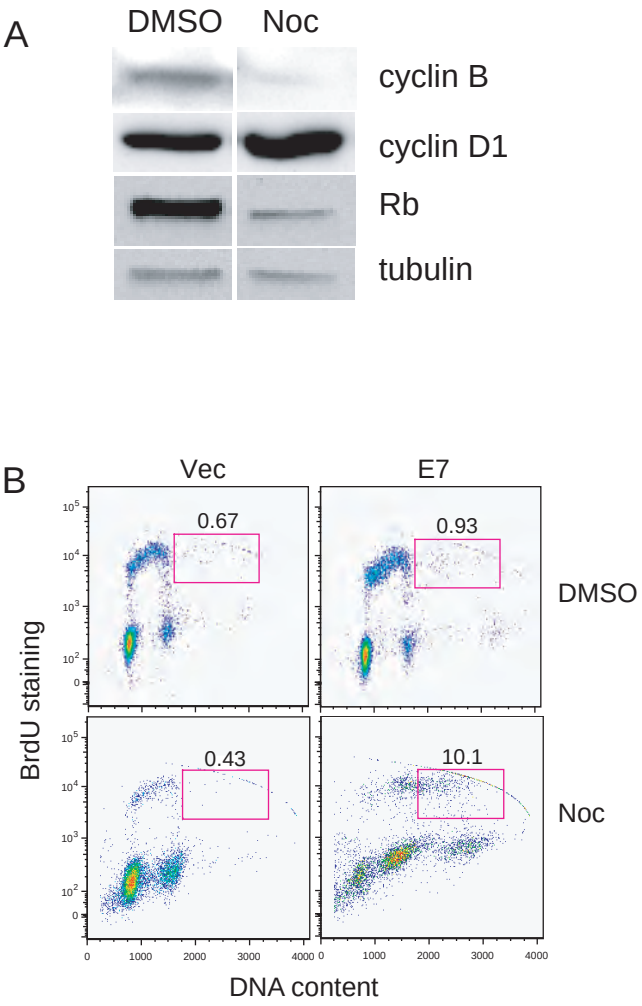
We first treated RPE1-vector cells with nocodazole for 48 hours in order to activate the spindle assembly checkpoint and obtain cells with 4C DNA content. We

then analyzed in which cell cycle stage these RPE1-vector cells were halted. We measured the protein expression levels of the G1 cyclin D1 and the G2 cyclin B and Rb phosphorylation. Figure 3.5A shows that in RPE1-vector cells, cyclin D1 was increased approximately 2-fold, cyclin B was decreased approximately 4-fold, and pRb became hypophosphorylated after nocodazole treatment. These results indicate that some of the RPE1 cells are halted in a G1 cell cycle state two days after nocodazole treatment.

Next, the extent to which E7 expressing RPE1 cells are capable of replicating DNA following adaptation to the spindle assembly checkpoint was tested. We incubated nocodazole-treated RPE1-vector and RPE1-E7 cells with BrdU and examined the ability of the cells with 4C DNA content in a G1 state to incorporate BrdU after they adapted to the spindle assembly checkpoint and exited mitosis. As shown in Figure 3.5B, 10% of RPE1-E7 cells with DNA content >4C incorporated BrdU, whereas only 0.4% of vector control cells with >4C DNA content incorporated BrdU. These results suggest that Rb-downregulation causes polyploidy in cells with 4C DNA content in a G1 state via abrogation of the postmitotic checkpoint.

Figure 3.5. Rb-downregulation in cells with a 4C DNA content in a G1-like state results in replication. A. RPE1 cells arrest in a G1-like state after nocodazole (50 ng/mL) treatment. Asynchronous cultures of RPE1-vector cells were incubated in nocodazole containing medium for 48 hours. Total protein extracts were resolved by SDS-PAGE and blotted with antibodies against cyclin B, cyclin D1, pRb, and tubulin. B. RPE1-vector and E7 expressing cells were treated with nocodazole or DMSO for a total of 72 hours. At 70 hours post treatment, the cells were incubated for the remaining 2 hours with BrdU. The ability of cells with 4C DNA content to incorporate BrdU was examined. BrdU positive cells with DNA content >4C are indicated. Data from a representative experiment (of 3) are shown.

Figure 3.5


A
DMSO
Noc
cyclin B
cyclin D1
Rb
tubulin
B
Vec
E7
0.67
0.93
0.43
10.1
DMSO
Noc
BrdU staining
DNA content
0
1000
2000
3000
4000

Discussion

This study explores the mechanism underlying the generation of polyploidy in Rb-downregulated cells. A number of possibilities was tested, and the data indicate that Rb does not have an effect on the spindle assembly checkpoint or on the length of time cells spent in mitosis. This is in contrast to Hernando et al (2004) who suggest that Rb downregulation and the subsequent effects on the Rb pathway, including upregulation of Mad2, result in a hyperactive spindle checkpoint (Hernando et al, 2004), which has been proposed to predispose cells to additional genomic instability. This discrepancy may be due to the interpretation of the time-lapse videomicroscopy results, which differs from theirs. They measured the length of mitosis from prometaphase to late anaphase by examining chromosome condensation and cell division (Hernando et al, 2004). On the other hand, I measured mitosis from NEB to NER, which are more distinctly defined and easily seen parameters. Also, in the previous study shRNA or expression of the oncogene E1A was used to downregulate Rb, both of which may affect genes other than Rb (Helt & Galloway, 2003; Hsieh et al, 2004; Kumar et al, 2003). Instead, the experiments in this study employed Rb-null MEFs, which completely lack any Rb, in addition to E7-expressing cells. A more recent study with Mad2 inducible upregulation demonstrates an increase in polyploid cells and a link to chromosomal instability upon Mad2 overexpression (Sotillo et al, 2007). However, cells overexpressing Mad2 do not appear to be equivalent to Rb-downregulated cells, as Mad2 overexpression led to partial mitotic block of proliferating cells, a phenomenon never seen in Rb-null cells.

The data also suggest that DNA re-replication is not a significant cause of polyploidization in Rb-downregulated cells in response to microtubule disruption.

Rather, I have shown that normal cells treated with low doses of nocodazole adapt to the spindle assembly checkpoint and exit mitosis. They then proceed into G1 with a 4C DNA content where they are stopped at the postmitotic checkpoint. Rb-downregulated cells proceed through this checkpoint, replicate their DNA, and become 8C. Thus, these results demonstrate that Rb is important for maintenance of the postmitotic checkpoint, and that Rb-downregulated cells are able to proceed through this checkpoint, replicate their DNA, and become polyploid.

This study used Western blot analysis of a minimal number of cell cycle markers, including cyclin B, cyclin D1, and phosphorylated Rb, to demonstrate that the cells are in a G1-like state. In order to more fully determine the cell cycle state that RPE1 cells are in after prolonged nocodazole treatment, a more detailed analysis of cell cycle markers may be used. Other cyclins may be checked including the G1/S phase transition cyclin E, the mitotic cyclin A, and the other G1 cyclins D2 and D3. A better test of cell cycle state would also be to perform kinase assays on the cdks associated with particular cyclins at various stages of the cell cycle. For example, if cells are in a G1-like state and Rb is hypophosphorylated, cdk2, 4, and 6 activity when associated with cyclin D should be decreased. In fact, it would be useful to analyze the cell cycle state and kinase activity at various time points after addition of nocodazole in both RPE1 and PHKs with or without E7 expression to study the kinetics of cell cycle proteins during nocodazole treatment.

As previously suggested and as the data here indicate, the postmitotic checkpoint is Rb-dependent (Lanni & Jacks, 1998). However, p53 also appears to play a key role in mediating the postmitotic checkpoint (Andreassen et al, 2001; Di Leonardo et al, 1997; Lanni & Jacks, 1998) and p21 is responsible for at least part of this p53-mediated

postmitotic arrest (Khan & Wahl, 1998; Lanni & Jacks, 1998; Stewart et al, 1999). The cdk inhibitor p21 binds to and inactivates cyclin/cdk complexes including cyclinE1/cdk2 and cyclinA2/cdk2, resulting in pRb hypophosphorylation that inhibits E2F activity and arrests the cell cycle at the G1/S transition (Stewart & Pietenpol, 2001; Vogelstein et al, 2000). Our lab has previously shown that p53 levels are not downregulated in E7 expressing cells. However, with pRb already down-regulated, the primary target of p53 and p21 at this checkpoint does not exist. Therefore polyploidization can occur in Rb-downregulated cells in the presence of an intact p53/p21 pathway.

This study has important implications for cancer in general, and for cervical cancer in particular, because of the results with the HPV oncogene E7. HPV E6 and E7 are the oncogenes necessary for transformation in high-risk HPVs, which are associated with cervical lesions that eventually may lead to cervical carcinomas. Although E6 and E7 are able to immortalize primary human epithelial cells, they are not sufficient to induce transformation of human cells (Fan & Chen, 2004). Instead, it is believed that genomic instability caused by E6 and E7 predisposes the cells to accumulate additional genomic aberrations necessary for malignant transformation (Duensing et al, 2000; Duensing & Munger, 2002b). As shown here and in other studies, HPV-E7 expressing cells are able to become polyploid (Patel et al, 2004; Thomas & Laimins, 1998; Thompson et al, 1997). Also, Olaharski et al (2006) concluded that polyploidy and chromosomal instability are related events that predispose cells to aneuploidy and subsequently to malignancy (Olaharski et al, 2006). The finding here that Rb-downregulation results in polyploidization through abrogation of the postmitotic checkpoint has important implications for cancer prevention and/or treatment.

Understanding this mechanism of polyploidization will be useful in developing therapies against its accumulation in Rb-downregulated, untransformed cells in order to prevent accumulation of additional genomic aberrations that could lead to carcinogenesis.

CHAPTER IV

CONCLUSIONS

The first half of this dissertation examined the effect of *Cbfb/MYH11* on leukemogenesis in the presence or absence of a wild-type *Cbfb* allele. In this study, transgenic mice that express the fusion gene *Cbfb/MYH11* on one allele and are *Cbfb* null for the second allele were created. These mice develop leukemia with a significantly shorter latency than those *Cbfb/MYH11* mice that retain a copy of wild-type *Cbfb*. Both genotypes of mice present with all of the characteristics of myeloid leukemia, including an increase in white blood cells, a decrease in red blood cells (anemia), and infiltration of the bone marrow, spleen and liver. Most importantly, flow cytometry analysis of peripheral blood from $Cbfb^{+/MYH11}$ and $Cbfb^{-/MYYH11}$ leukemic mice shows a block in the differentiation of hematopoietic cells including decreases in the percentages of granulocytes, macrophages, T-cells, and B-cells and an increase in the percentage of c-kit^{+} progenitor cells.

Pre-leukemic analysis of the bone marrow cells from both *Cbfb/MYH11*-expressing mice supports a block in differentiation as well. Flow cytometry analysis of these pre-leukemic cells shows an increase in the number of hematopoietic stem cells, and also an increase in myeloid progenitor cells. These progenitor cells show immuno-phenotypic characteristics of MEPs, but the peripheral blood smears show cells with myelo-, mono-, and erythro-blast morphologies, and the *in vitro* assay confirms that these progenitors have the differentiation capacity of CMPs—they are able to form BFU-E, CFU-GM, and CFU-GEMM colonies. These cells look similar to the population previously shown by Kuo et al (2006) called abnormal myeloid progenitors (AMPs) (Kuo

et al, 2006). These AMP-like cells are the precursors that will expand into the fully transformed leukemic cells.

Leukemia, simply, is caused by both a block in differentiation of hematopoietic cells and an increase in proliferation of these cells. The fusion protein Cbfβ-SMMHC causes a partial block in differentiation, and it is theorized that a second, cooperating mutation conveying a proliferative advantage is needed for full transformation. In general, mice acquire random genetic mutations over time. Subsequently, after a long enough period of time, mice with expression of *Cbfb/MYH11* may acquire a genetic mutation in a gene that provides a proliferative advantage to the cell. This cell expands to cause leukemia from a clonal population of transformed cells. Because the *Mx1;Cre* system is "leaky" there is some minimal expression of the fusion gene without induction by pIpC. This event is sufficient to cause expansion of cells containing *Cbfb/MYH11* and a null *Cbfb*. In this study, there was a significantly shorter latency in *Cbfb/MYH11* mice without wild-type *Cbfb* compared to those mice expressing it.

The short latency in the $Cbfb^{-/MYH11}$ mice suggests one of two possible scenarios. The first is that there are no acquired mutations because there has not been adequate time in which to attain them. In this case, the two mutations are sufficient-- the presence of *Cbfb/MYH11* causes a block in differentiation and the lack of *Cbfb* creates a dominant effect, not just inhibiting normal hematopoiesis, but also adding a transforming effect to this expanding progenitor cell population. This possibility could be analyzed using a conditional $Cbfb^{-/-}$ mouse. Normally $Cbfb^{-/-}$ mice are embryonic lethal due to a lack of definitive hematopoiesis as was seen in the *Cbfb/MYH11* heterozygous mice. Creation of a conditional *Cbfb* knock-out mouse model would allow normal development to

adulthood with both *Cbfb* alleles expressed but flanked by Lox-P sites. These mice would be crossed with *Mx1;Cre* mice, and the second *Cbfb* allele could be deleted in adulthood. First, these mice could be monitored for onset of leukemia, in order to determine if the lack of wild-type *Cbfb* is sufficient for leukemogenesis. Our previous studies suggest that *Cbfb/MYH11* is necessary for transformation, although it is possible that the lack of *Cbfb* may induce a myeloproliferative disease instead of cancer. My experiments and those from Kuo et al (2005) show that it is the presence of the fusion gene that dictates the phenotype of the leukemia. If these $Cbfb^{-/-}$ mice fail to develop leukemia, or if they develop leukemia with a long latency, the *Cbfb-MYH11* fusion gene could be introduced by retroviral infection to bone marrow from these mice and then injected into wild-type irradiated recipients. This approach would result in mice whose bone marrow cells contained the fusion protein, but no copies of wild-type Cbfb. These mice would then also be monitored for onset of leukemia. These mice would be expected to become leukemic, because this genotype essentially recapitulates that of the $Cbfb^{-/MYH11}$ mice. However, experiments with the conditional *Cbfb* knock-out mice could be used to address questions about the latency of leukemia between the two mouse models. These mice could also be used to test whether the temporal occurrence of the two mutations is important.

The second possible explanation for the short latency in the *Cbfb-/MYH11* mice is that the complete block in differentiation caused by both the presence of *Cbfb/MYH11* and the lack of wild-type *Cbfb* causes a dramatic expansion in the progenitor population that significantly increases the likelihood and decreases the latency of the occurrence of additional mutations. It has generally been observed that cells that differentiate no longer

proliferate and vice versa. Therefore it has been suggested that decreased differentiation may act as a proliferative stimulus (Cao et al, 1997) or that there will at least be some compensatory increase in the number of progenitor cells from a decrease in differentiation (Kundu & Liu, 2003). In the *Cbfb*$^{-/MYH11}$ mice, the increase in progenitor cells most likely provides a larger more susceptible pool of cells that are prone to acquiring proliferative mutations. The full block in differentiation from both *Cbfb* mutations may cause acceleration of this natural acquisition of genetic mutations.

Potential proliferative mutations include those that are able to induce S-phase progression. One study showed that mice with bone marrow cells expressing both CBFβ-SMMHC and the HPV-16 E7 oncogene developed leukemia faster than those mice with only CBFβ-SMMHC. In these cells, E7 binds to and degrades Rb so that the E2F transcription factors are free to activate S-phase proteins. This significantly increases the rate of proliferation in these cells under normal conditions and under certain mitotic stresses. The latter half of this dissertation explores the possible mechanisms through which E7 is able to cause cell cycle progression and subsequently polyploidization.

Affecting 6% of women worldwide, cervical cancer is one of the most common cancers in the world (Parkin et al, 2001). Although there is now a prophylactic vaccine for the two most common high-risk types of HPV, cervical cancer research must continue because the vaccine does not affect currently infected individuals or the immuno-compromised, nor does it prevent all types of HPV infection.

Although a high-risk HPV genome is present in greater than 99% of cervical cancer cases, this does not necessarily induce nor guarantee transformation. Only a fraction of high-risk HPV infected cells become cancerous, and it often occurs after a

significant latency. This suggests that HPV is not sufficient for transformation, but that the virus (and presumably its oncogenes) predisposes the infected cells to accumulate additional genomic mutations that will then lead to cancer.

It has previously been shown that while the HPV oncogene E7 is able to immortalize cells, it is not sufficient for transformation. However E7 has been shown to induce genomic instability, a precursor to polyploidy. While the genetic mutations that cause polyploidy may not be sufficient for transformation alone, the accumulation of polyploid cells results in aneuploidy, and genomic instability, which increases the probability of acquiring additional mutations (Duensing et al, 2000; Duensing & Munger, 2002b). In the case of HPV E7, once these numerical alterations occur, continued expression of the oncogene is still necessary to maintain transformation (Doorbar 2006). The method by which E7 induces polyploidy may be used to develop therapies against induction of cervical cancer. Several conflicting mechanisms to polyploidization have been suggested for E7 expressing, Rb-downregulated cells. This study explored each of these proposals in order to better understand how polyploidization occurs in these cells.

This study shows that Rb-downregulation does not play a role in mitosis, either under normal conditions or upon microtubule disruption. Although Rb may play some role in cytokinesis as seen with the increased length of cytokinesis in the time-lapse data, the downregulation of Rb did not prevent abscission from occurring. The time-lapse videomicroscopy also did not show the presence of any fused cells. Because cellular fusion usually occurs with viral infection, it may require additional viral proteins besides E7.

Re-replication is a process where the cell completes S-phase replication and fails to enter or complete mitosis before replicating the DNA again. It may also be described as the process where DNA is replicated two or more times without completed cell division. However, the preferred definition makes the distinction that re-replication does not occur if the cells have at least entered mitosis, even if it is not completed. Therefore, this study shows that Rb-downregulated cells do enter mitosis, although the process is not completed before the cells re-enter S-phase.

Instead, after cells fail to complete mitosis due to microtubule disruption, they proceed into a G1-like state as determined by protein expression levels of cyclins and phosphorylated Rb. However, in this case wild-type cells have a 4N DNA content and are normally arrested in this state. The precise trigger for this post-mitotic checkpoint remains unknown, but it seems logical for the cell to have a checkpoint that protects against replication of 4N cells, which could lead to proliferation of polyploid cells. In HPV-16 E7 expressing cells, E7 binds to and causes degradation of Rb, which releases the E2F transcription factors to activate S-phase proteins. Thus, E7-expressing, Rb-downregulated cells replicate their 4N genomes leading to 8C polyploid cells.

These findings in $Rb^{-/-}$ MEFs and Rb-downregulated cells would suggest that HPV-E7 abrogation of the postmitotic checkpoint is Rb-dependent. However, E7 does have Rb-independent activities, including inhibiting the activity of p21 and p27 (Funk et al, 1997; Jones et al, 1997; Zerfass-Thome et al, 1996) which affect their cognate cdk/cyclin complexes and may also abrogate cell cycle checkpoints in G1, S, and mitosis. In order to determine whether abrogation of the postmitotic checkpoint by HPV-E7 is Rb-dependent, mutation studies involving E7 mutants deficient for degradation and/or

binding of Rb will be necessary. There are several known mutants that may be useful. Mutants Δ6-10, L67R, and LL82-83RR, retain binding to Rb but do not induce degradation. Mutants C24G, Δ21-24, and E26G neither bind nor degrade Rb. Based on the results of this study, it may be hypothesized that abrogation of the postmitotic checkpoint is an Rb-dependent activity of E7. Many of these E7 mutants are also defective for other E7 activities including inhibition of p21 and p27, and activation of cdc25A. Assessing the ability of these mutants to become polyploid upon microtubule disruption, will determine the importance of these other E7 functions in abrogation of the post-mitotic checkpoint and polyplpoidization.

E7 induced polyploidization should also be studied in the context of both E6 and the entire HPV genome. Expression of HPV-16 E6 also causes abrogation of the post-mitotic checkpoint upon microtubule disruption, and combined expression of E6 and E7 leads to an increase in the number of polyploid cells in a population (Liu et al, 2007). Therefore, expression of not just E7 but also the entire HPV genome may also lead to an increase in polyploidization. However, E2 has been shown to negatively regulate E7, which may cause a decrease in polyploidy. Also, E4 has recently been shown to increase re-replication (Knight et al, 2004), which may also lead to polyploidy and result in an increase in polyploid cells upon expression of the HPV genome.

Both of the studies presented in this dissertation examine the role of a chromosomal abnormality in carcinogenesis. The structural chromosomal aberration *Cbfb*/*MYH11* is necessary but not sufficient for leukemogenesis. However, the lack of wild-type *Cbfb* in these cells leads to the accumulation of cooperating mutations and

subsequently AML. The protein E7 is also insufficient for transformation in HPV positive cells. However, the expression of E7 does result in polyploidization, a numerical chromosomal aberration that leads to aneuploidy and carcinogenesis through accumulation of additional genetic mutations.

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